APPENDIX D

1. Hellstrom et al. (2001), *Proc. Natl. Acad. Sci. USA* **98**(12):6783-88;

2. Orchard, P.J. et al. (2002) *Hum Gene Ther.* **13**(8):979-88;

3. Pene J. et al. (2003) *J. Immunol. Methods* **283**:59-66; and

CD3-mediated activation of tumor-reactive lymphocytes from patients with advanced cancer

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Lymphocytes from blood or tumors of patients with advanced cancer did not proliferate and produced very low levels of tumor necrosis factor and IFN-γ when cultured with autologous tumor cells. Proliferation and lymphokine production dramatically increased in the presence of beads conjugated with mAbs to CD3 plus mAbs to CD28 and/or CD40, and the lymphocytes destroyed the tumor cells. Expression density of CD3 concomitantly increased from low to normal levels. Furthermore, beads providing a CD3 signal (in combination with CD28 or CD28 plus CD40) gave partial protection against the inhibitory effect of transforming growth factor type β1 on lymphocyte proliferation and production of tumor necrosis factor and IFN-γ. MHC class I-restricted cytolytic T cells lysing autologous tumor cells in a 4-h Cr^{51} release assay were generated when peripheral blood leukocytes were activated in the presence of autologous tumor cells and anti-CD3/CD28 or anti-CD3/CD28/CD40 beads. Experiments performed in a model system using anti-V-β1 or anti-V-β2 mAbs to activate subsets of T cells expressing restricted T cell receptor showed that lymphocytes previously activated by anti-V-β can respond to CD3 stimulation with vigorous proliferation and lymphokine production while retaining their specificity, also in the presence of transforming growth factor type β1. Our results suggest that T lymphocytes from cancer patients can proliferate and form Th1 type lymphokines in the presence of autologous tumor cell when properly activated, and that antigen released from killed tumor cells and presented by antigen-presenting cells in the cultures facilitates the selective expansion of tumor-directed, $CD8^+$ cytolytic T cells.

immunotherapy | tumor vaccines | tumor immunity | transforming growth factor type β1

Immune responses do not protect against most cancers, although data were published in the 1960s indicating that the immune systems of cancer patients recognize antigens that can be targets for tumor destruction (1). Recent evidence for immunogenic, tumor-associated antigens includes the demonstration, with tetramer technology, that lymphocytes from melanoma patients recognize tumor epitopes (2), the finding of IgG antitumor antibodies by using the SEREX technique (3), and the generation of cytolytic T cells (CTL) to a large variety of tumor epitopes (4, 5). Most likely, the failure of immunological mechanisms to prevent tumor formation is due to mechanisms normally protecting against autoimmunity. For example, most neoplastic (like most normal) cells do not express key costimulatory molecules and are able to "sneak through" immunological control until their antigens have been taken up and processed by "professional" antigen-presenting cells (6–8). Furthermore, tumors make immunosuppressive factors, as can lymphoid cells in response to tumor antigens (9). Members of the transforming growth factor (TGF) type β1 family (9–15) are particularly important in this regard. Reflecting the immunosuppressed state, molecules involved in T cell signaling are down-regulated among lymphocytes from blood or tumors derived from tumor-bearing animals and human patients (16–18). Unless the immunosuppression can be overcome, it is unlikely that tumor vaccination or adoptive transfer of immune T lymphocytes will have a major impact in patients with metastatic cancer.

We now show that lymphocytes from patients with advanced cancer can proliferate, produce high levels of tumor necrosis factor (TNF) and IFN-γ, and generate tumor-destructive CTL in the presence of autologous tumor cells after polyclonal activation, via CD3, and costimulatory signal(s), via CD28, alone or together with CD40. We further show that lymphocytes stimulated via CD3 plus costimulatory signals become relatively resistant to inhibition by TGF-β1. These data are supported by experiments performed in a model system where anti-V-β1 and anti-V-β2 are used as surrogate antigens to which responses are induced or recalled by using the respective specific mAbs.

Materials and Methods

Patient Material. Tumors were obtained at surgery or from malignant effusions (mostly ascites) of patients with stage IV carcinomas. Most studies were performed with eight patients, five of whom (1OV, 3OV, 8OV, 44OV, 48OV) had ovarian carcinoma, two (1C, 22C) had colon carcinoma, and one (1HN) had a head and neck carcinoma. Cells from an ovarian carcinoma line, 4007, also were used.

Preparation of Tumor and Blood Samples. Solid tumors were suspended in medium, and fluids were removed from effusions after which the cells were resuspended. Erythrocytes were removed by Ficoll-Hypaque (Amersham Pharmacia), and a Percoll gradient (Sigma) was used to separate tumor cells from tumor-infiltrating lymphocytes (TIL). Lymphocyte samples were used directly or stored in liquid nitrogen for later use. Tumor samples were explanted to establish cell cultures. Peripheral blood leukocytes (PBL) were purified by using Ficoll-Hypaque. In the initial experiments, $CD8^+$ T lymphocytes (>90% pure) were positively selected from TIL by using VarioMac magnetic beads (Miltenyi Biotech, Auburn, CA). For all other experiments, lymphoid cell populations containing T lymphocytes, monocytes, and B cells were used.

Combination of Lymphocytes and Tumor Cells. In the initial experiments, five lymphocytes were added per tumor cell, after which the mixtures were incubated at 37°C in Costar (3513) 12-well plates (Corning) with RPMI medium (GIBCO) and 10% FCS (Atlanta Biological, Norcross, GA). They were followed by experiments in which PBL or TIL were cultured with or without autologous tumor cells in the presence of magnetic beads (Dynal, Lake Success, NY) and conjugated, using a published technique (19, 20), with mAbs to CD3, CD28, and/or CD40; beads not conjugated with mAb (or

Abbreviations: CTL, cytolytic T cells; FACS, fluorescence-activated cell sorting; PBL, peripheral blood leukocytes; TGF, transforming growth factor; TIL, tumor-infiltrating lymphocytes; TNF, tumor necrosis factor.

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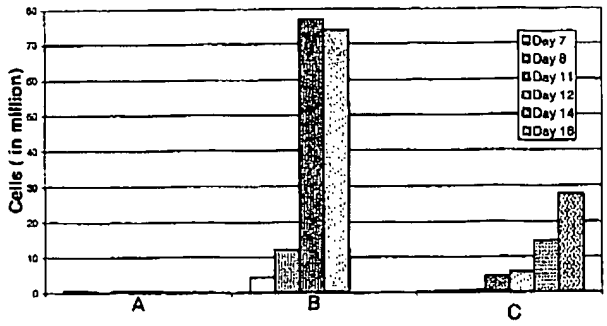

Fig. 1. Proliferation of *in vitro* expanded TILs from 44 OV. (*A*) Autologous tumor, control beads (similar results without tumor). (*B*) Autologous tumor, anti-CD3/CD28/CD40-conjugated beads. (*C*) No tumor, anti-CD3/CD28/CD40-conjugated beads.

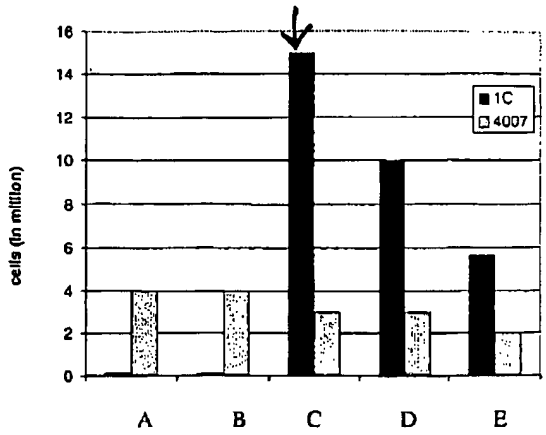

Fig. 2. Proliferation of PBL from 1C after *in vitro* activation in the presence of autologous or allogeneic 4007 mismatched ovarian carcinoma cells. (*A*) Control beads. (*B*) Anti-CD28/CD40-conjugated beads. (*C*) Anti-CD3/CD28-conjugated beads. (*D*) Anti-CD3/CD28/CD40-conjugated beads. (*E*) Anti-CD3/CD40-conjugated beads.

with an irrelevant mAb) were used as controls. The mAbs were 64.1 (21), 9.3 (21), and G28.5 (22), which, respectively, stimulate lymphocytes polyclonally (anti-CD3), costimulate them (anti-CD28), or activate antigen-presenting cells (anti-CD40). When autologous tumor cells were used, cells (40,000–75,000/well) were first attached by overnight incubation to Costar 24-well plates containing 2 ml Iscove's modified Dulbecco medium with 10% FBS. mAb-conjugated beads (3×10^6/ml) were then added, followed by lymphocytes (10^6/ml) in RPMI with 10% FBS. The plates were incubated at 37°C in a 6% CO_2 in air atmosphere for 4–5 days. The beads then were removed using a magnet, and the lymphocytes were placed in new wells in medium containing 10 units/ml of IL-2 (Roche Molecular Biochemicals) and moved into flasks when their concentration had reached $\approx 2 \times 10^6$ cells/ml. Cultures were observed for evidence of tumor cell destruction. Lymphocyte proliferation was determined by counting. Media were sampled to measure production of TNF and/or IFN-γ, which was assayed with WEHI cells (23) and an ELISA (IFN-γ ELISA, EH-IFNG, Endogen, Woburn, MA), respectively. TGF-β1 was purchased from Sigma. In all experiments using TGF-β1, the molecule remained in the cultures, also after removal of mAb-conjugated beads.

CTL Assays. Classical 4-h ^{51}Cr release assays were performed. To characterize the effector cells, experiments were done to inhibit cytotoxicity by addition of mAb w6/32 (10 μg/ml), which recognizes a MHC class I framework determinant (Research Diagnostics, Flanders, NJ). mAbs to the natural killer markers CD16 and CD56 (Beckman Coulter), anti-CD8 mAb HIT8a (PharMingen), and anti-integrin-β2 (CD18) mAb 60.3 (24) also were used.

Fluorescence-Activated Cell Sorting (FACS) Analysis of Lymphocytes. Density of CD expression was evaluated by FACS (Epics XL, Coulter), using phycoerythrin-labeled mAb and counting cells as positive when they had a preset minimum brightness. To investigate whether an increased density of CD3 expression after *in vitro* activation of lymphocytes was due to the selective proliferation of cells with originally high CD3 expression, PBL harvested from cancer patients were labeled with the dye CFDA (Molecular Probes). Subsequently, they were cultured in the presence of anti-CD3/CD28/CD40 beads for 5 days, after which the beads were removed and the lymphocytes were expanded in medium containing 10 units IL-2/ml. At two time points after removal of the beads (4 h and 3 days) FACS analysis was performed, in which cells were analyzed for labeling by CFDA and expression of CD3. Labeled lymphocytes that had been cultured with control beads were studied for comparison.

Use of a Model System with Anti-V-β1 and Anti-V-β2 as Surrogate Antigens. Experiments were performed to investigate the effect of stimulation of PBL from healthy adult donors (one donor in each experiment) on the proliferation and lymphokine production of naïve lymphocytes and on lymphocytes activated by mAbs to V-β1 and V-β2 (Beckman Coulter). The well bottoms of a culture plate were coated with 1 μg/ml of anti-V-β1 or anti-V-β2 by incubation at 4°C overnight. Subsequently, PBL (2×10^6/well) were added, either together with control beads or beads conjugated with anti-CD3/anti-CD28/anti-CD40 mAbs. After 4–5 days of coculture, beads were removed and lymphocytes were expanded in medium supplemented with 10 units IL-2/ml. The number of lymphocytes/well was counted, and production of TNF and IFN-γ was measured. Lymphocytes were analyzed by FACS for expression of CD3, V-β1, and V-β2.

A second round of stimulation then was carried out to study the effects of stimulation by anti-V-β and/or mAb-coated beads on sensitized lymphocytes. In these experiments, lymphocytes sensitized in the presence of either anti-V-β were cultured for 5 days in the presence of the same or a different, immobilized anti-V-β, with or without beads conjugated with anti-CD3/CD28/CD40 mAbs.

Results

Cocultivation of PBL or TIL with Autologous Tumor Cells. Six initial experiments were performed in which $CD8^+$ T lymphocytes purified from TIL were cultured with tumor cells, after which the supernatants were assayed for TNF or IFN-γ. In a representative experiment, $CD8^+$ TIL from a colon cancer patient (1C), first cultivated with 1C tumor cells for 15 days, were removed and added to either a fresh set of 1C cells or to tumor cells from a lung carcinoma patient (3L). A small amount of TNF (1.2 pg/ml) was detected when 1C lymphocytes were combined with the 1C but not with the 3L tumor, whereas TNF and IFN-γ (1.5 pg/ml) were produced when TIL from 3L were combined with 3L tumor cells but not when cultured alone. There was no evidence of lymphocyte proliferation. In subsequent experiments, TIL populations comprising monocytes, $CD4^+$ T cells, and B cells in addition to $CD8^+$ lymphocytes were combined with autologous tumor cells. Approximately 10 times higher levels of TNF (4.5–48 pg/ml) and up to 150 pg/ml of IFN then were detected in supernatants from cultures of eight of 13 patients. There was still no lymphocyte proliferation.

We then adapted a system in which mAb-conjugated magnetic beads are used to induce signals via various lymphocyte receptors (19, 20). PBL or TIL were combined with autologous tumor cells in the presence of beads conjugated with mAbs to CD3 and mAbs to CD28, alone or together with CD40. Similar groups were included with lymphocytes but without tumor cells. As controls,

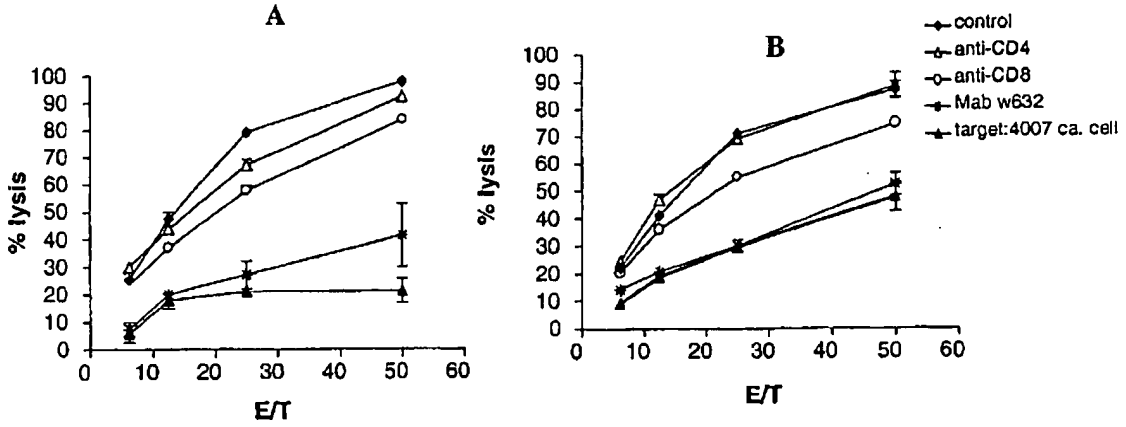

Fig. 3. Cr^{51} release data with PBL from 1C, tested on the indicated target cells, after activation on 1C cells by anti-CD3/CD28-conjugated beads (*A*) or anti-CD3/CD28/CD40-conjugated beads (*B*). Standard deviations were <10% of cpm. E/T, effector-to-target cell ratio.

lymphocytes, with or without tumor cells, were cultivated with control beads. After 3–5 days, the beads were removed and the lymphocytes were incubated separately over 2–21 days with 10 units/ml of IL-2.

Fig. 1 shows an experiment in which TIL from OV44 proliferated vigorously when exposed for 4 days to anti-CD3/CD28/CD40-conjugated beads. Lymphocytes cultivated in the absence of a CD3 signal did not proliferate and neither did lymphocytes cultured with anti-CD28 and/or CD40 beads (data not shown). Proliferation was greater when autologous tumor cells were initially present with the beads inducing signals via CD3 (Fig. 1*B*). Anti-CD3/CD28-conjugated beads induced proliferation similar to that with anti-CD3/CD28/CD40-conjugated beads (data not shown).

Fig. 2 shows an experiment in which PBL from patient 1C and various mAb-conjugated beads were cultivated for 5 days with either autologous tumor cells or allogeneic (4007) cells. The number of lymphocytes per culture was much higher when CD3/CD28 (Fig. 2*C*) or anti-CD3/CD28/CD40 (Fig. 2*D*) activated lymphocytes were combined with 1C tumor than with 4007 cells, a finding similar to that illustrated in Fig. 1. When, on the other hand, the beads did not provide any signal via CD3 (Fig. 2 *A* and *B*), the situation was the reverse and probably represented an immunological response to alloantigens expressed on the 4007 cells. FACS analysis showed that >90% of the activated lymphocytes expressed CD3 and ≈70% of them were $CD8^+$, with less than 5% expressing CD16 or CD56.

Most of the tumor cells were destroyed within 24–48 h after exposure to autologous lymphocytes in the presence of anti-CD3/CD28- or anti-CD3/CD28/CD40-conjugated beads, often leaving cultures entirely comprising cells with lymphocyte morphology. To study whether this destruction had immunological specificity, four experiments were performed in which serial dilution of PBL (10^6–10^5/sample) from cancer patients were combined with autologous tumor cells or with either tumor cells or fibroblasts from an allogeneic donor. In two experiments, there was ≈10 times more TNF in the culture supernatants in the presence of the autologous tumor, but there was no difference in the killing of cells from autologous or allogeneic tumors or of allogeneic fibroblasts. We conclude that tumor cell destruction seen after 24–72 h in the presence of lymphocyte activation was not immunologically specific, perhaps because large amounts of activated T lymphocytes and lymphokines obscured any specific components.

MHC-class I-restricted CTL were generated from lymphocytes activated by tumor cells plus anti-CD3/CD28 or anti-CD3/CD28/CD40 beads. Fig. 3 presents an experiment with PBL from 1C, which had been activated in the experiment shown in Fig. 2. After activation by tumor cells and mAb-conjugated beads, the beads were removed and the lymphocytes were expanded with 10 units IL-2/ml medium over 3 weeks in the absence of tumor cells and beads. PBL activated by 1C and anti-CD3/CD28 beads were strongly cytolytic to 1C cells, and lysis was inhibited by a mAb to CD8 and by anti-MHC class I framework mAb w6/32 (Fig. 2*A*). Allogeneic 4007 cells were killed by only 20% at an effector-to-target cell ratio of 50:1, as compared with 98% lysis of 1C cells (Fig. 2*A*). Fig. 2*B* demonstrates analogous data for PBL stimulated with anti-CD3/CD28/CD40 beads. Lysis of 4007 cells then was at the same low level as that of 1C in the presence of mAb w6/32. In contrast, PBL stimulated with anti-CD3/CD40 beads killed both 1C and 4007 cells, also in the presence of mAbs to CD8 or mAb w6/32 (data not shown). $CD8^+$ cells enriched from the cell population used in the experiment shown in Fig. 2*B* lysed 25% of 1C cells at an effector-to-target cell ratio of 1:20 as compared with 0% of cells from the 4007 line and 0% of cells from an allogeneic B cell line. In this experiment, lysis of 1C cells was 5% in the presence of mAb w6/32 and 5% with the anti-CD18 mAb 60.3, and it only decreased from 25% to 18% with a combination of mAbs to CD16 and-CD56. Lymphocytes activated by cocultivation with 4007 cells and any of the beads did not selectively lyse 1C or 4007 cells (data not shown). The CTL assays were repeated twice with similar results.

Large amounts of IFN-γ were detected in supernatants of cultures from lymphocytes activated via CD3 (Fig. 4). Fig. 4 also shows that the production of IFN-γ was higher when autologous tumor cells were present during the first 4–5 days of culture.

Table 1 presents six additional representative experiments show-

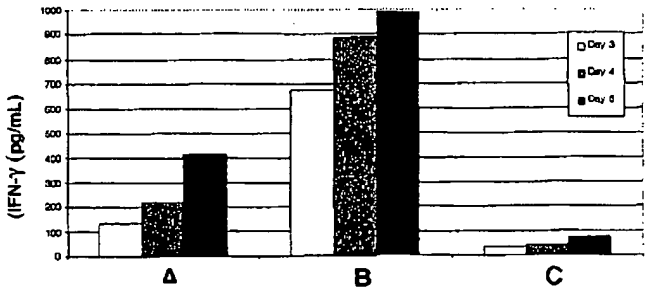

Fig. 4. IFN-γ produced by *in vitro* expanded PBL from 1HN. (*A*) No tumor, anti-CD3/CD28-conjugated beads. (*B*) Autologous tumor, anti-CD3/CD28-conjugated beads. (*C*) Autologous tumor, control beads (similar results without tumor).

Table 1. Proliferation (cell numbers × 10^6 per sample) and lymphokine production (pg/ml of TNF or IFN-γ) of freshly harvested PBL and TIL from patients with advanced cancer after culturing for 4–5 days ± autologous tumor cells in the presence of mAb-conjugated beads, followed by 2–4 days without beads (same time within each experiment)

mAb-conjugated beads	PBL								TIL				
	10V			80V		1HN*		480V*	30V			22C*	
	× 10^6	TNF	IFN-γ	× 10^6	TNF	× 10^6	TNF	× 10^6	× 10^6	TNF	IFN-γ	× 10^6	TNF
Control	1.1	30	479	4.2	12	1.3	3	0.4	1.2	0	271	1.4	5
Anti-CD3	11.3	2,440	5,560	15.3	1,550	4.8	2,080	NT	8.2	950	3,720	NT	NT
Anti-CD3/CD28	9.6	2,500	7,810	22.7	2,500	7.2	13,020	NT	7.0	2,100	4,730	5.8	1,660
Anti-CD3/CD40	7.8	2,500	4,480	18.6	>1,000	4.0	1,450	NT	5.4	660	3,060	3.6	1,540
Anti-CD3/CD28/CD40	NT	NT	NT	NT	NT	NT	NT	17.1	NT	NT	NT	6.5	2,060

Cultures were initiated with 10^6 PBL or TIL/sample. NT, not tested.
*Autologous tumor cells present together with the lymphocytes.

ing proliferation and lymphokine production by PBL or TIL, which were either tested upon harvest from the patients or after one round of *in vitro* activation with beads. Anti-CD3, anti-CD3/CD28, anti-CD3/CD40, and anti-CD3/CD28/CD40 beads strongly increased lymphocyte proliferation with no significant difference between them. In contrast, anti-CD28, anti-CD40, and anti-CD28/CD40 beads alone did not increase lymphocyte proliferation and lymphokine production over control beads (data not shown), indicating that signaling via CD3 was essential. Production of TNF and IFN-γ correlated well. It decreased to background levels when the lymphocytes were grown without tumor cells and beads for more than 3–5 days (data not shown). As in Figs. 1 and 4, CD3 signaling was required to induce vigorous lymphocyte proliferation and lymphokine production.

The density of CD antigen expression on lymphocyte populations was measured by FACS before and after 3- to 5-day cultivation with tumor cells and anti-CD3/CD28/CD40 beads, followed by an additional 3- to 7-day expansion without beads. To reflect changes in the density of CD receptor expression, the number of cells in each population whose brightness at least equaled the density at the chosen setting is reported (Table 2); unstimulated PBL from six healthy donors (30–65 years of age) were analyzed for comparison. Unstimulated PBL from the cancer patients had low levels of CD3, CD4 and CD28. Four of five patients also had low CD8 density, whereas the CD86 density was higher than among unstimulated PBL from the healthy donors. Culturing of PBL with control beads partially increased CD3 expression, but did not significantly increase CD28 expression. In contrast, culturing with anti-CD3/CD28/CD40 beads consistently restored the expression of CD3 and CD28 to normal levels, and it doubled the number of cells with high-density CD8 expression. Density of CD3 expression was studied with TIL from five patients. It was 2.9%, 40.2%, 96%, 42.8%, and 40.1%, respectively—i.e., it displayed more variation and was generally higher than for PBL. CD8 expression by TIL was higher than among PBL and increased from 61.4% to 87.3%. The corresponding figures for CD28 expression among TIL were 39.3% and 52.8%. Before cultivation, PBL and TIL from cancer patients comprised 10–20% $CD14^+$ cells (most likely monocytes). At 2 to 3 days after cultivation with anti-CD3/CD28 or anti-CD3/CD28/CD40 beads, 5–20% of the lymphoid cells were $CD83^+/CD3^-$; there was less than 1% of such cells with control beads or anti-CD28/CD40 beads.

To investigate whether an increased density of CD3 expression after *in vitro* activation of lymphocytes was due to the selective proliferation of cells with originally high CD3 expression, experiments were performed with TIL, 40.2% of which originally expressed CD3, which were labeled with the dye CFDA (25). After activation via anti-CD3/CD28/CD40 beads, CD3 expression increased to 95%. FACS analyses, using CFDA and phycoerythrin-labeled anti-CD3 as probes, showed that there was no selective proliferation of the subpopulation of PBL that originally had higher CD3 expression.

Effect of TGF-β1 in the Presence of Activation Signals via mAb-Conjugated Beads. Table 3 shows five representative experiments performed to investigate whether the inhibitory effect of TGF-β1 on lymphokine production and lymphocyte proliferation could be altered by coculture with beads inducing signals via CD3. With control beads, the TNF and IFN-γ levels were low, and these levels were further suppressed by TGF-β1. In contrast, with anti-CD3/CD28/CD40 beads these levels increased to levels often approaching those seen in the absence of TGF-β1. Likewise, when

Table 2. CD expression (mean ± SD) of PBL from six healthy adults and from cancer patients (5–8 patients per group), tested directly (unstimulated) or after culturing with mAb-conjugated beads for 4–5 days

Marker	Healthy donors, unstimulated	Cancer patients				
			Beads			
		Unstimulated	Control	Anti-CD3/CD28	Anti-CD3/CD40	Anti-CD3/CD28/CD40
CD3	72.3 ± 11	20 ± 24*	52 ± 32	92 ± 10†	96 ± 5†	94 ± 5†
CD4	45.4 ± 11	21 ± 20	37 ± 24	42 ± 23	29 ± 20	51 ± 18
CD8	21.7 ± 10	9 ± 7	18 ± 13	47 ± 26	70 ± 15†	45 ± 15†
CD28	62.1 ± 12	33 ± 17*	45 ± 32	79 ± 30	70 ± 36	93 ± 3†
CD56	2.2 ± 3	11 ± 12	25 ± 34	2 ± 4	3 ± 2	1.5 ± 2.4
CD80	0.1 ± 0	2 ± 2	4	1 ± 1	3 ± 5	5.6 ± 9
CD86	0.2 ± 0	34 ± 24*	10 ± 13	10 ± 14	16 ± 16	5 ± 3

Five to eight samples were tested per group of cancer patients.
*, $P < 0.01$ compared to unstimulated lymphocytes from healthy donors.
†, $P < 0.01$ compared to unstimulated lymphocytes from patients.

Table 3. Proliferation (cell numbers × 10^6 per sample) and lymphokine production (pg/ml of TNF or IFN-γ) by fresh or previously stimulated (*) PBL and TIL from cancer patients cultured for 4–5 days with tumor cells and mAb-conjugated beads ± (5 ng/ml) TGF-β1, followed by 2–3 days without beads or tumor cells but with TGF-β1 remaining

mAb-conjugated beads	TGF-β1 present	PBL								TIL		
		22C			480V		1HN*			480V		22C*
		× 10^6	TNF	IFN-γ	× 10^6	IFN-γ	× 10^6	TNF	IFN-γ	× 10^6	IFN-γ	× 10^6
Control	−	4.6	40	264	0.5	310	1.3	3	23	4.4	98	1.7
	+	2.8	0	19	0.5	19	0.4	5	48	2.8	27	1.4
Anti-CD3/CD28/CD40	−	19.7	2,900	>20,000	17.2	>10,000	7.2	11,680	24,050	8.1	9,810	6.5
	+	15.5	860	6,700	9.8	3,810	7.4	4,640	19,250	3.7	5,220	3.1

Cultures were initiated with 10^6 PBL or TIL/sample.

anti-CD3/CD28/CD40 beads were used, there was much less inhibitory effect of TGF-β1 on lymphocyte proliferation with no inhibition at all seen with patient 1HN. A relative resistance of T cell proliferation and lymphokine production was seen also when the TGF-β1 dose was increased to 20 ng/ml and when the concentration of lymphocytes was decreased to 10^5/sample (data not shown). Beads stimulating via CD28, CD40, alone or together, did not protect against TGF-β1 (data not shown).

Model Experiments with mAbs to V-β1 and V-β2 as Surrogate Antigens. Because stimulation via CD3 interferes with the induction of primary immune responses, experiments were performed in a model system to investigate the effect of CD3 stimulation on lymphocytes that had been activated by an antigen. As surrogate antigen, we used mAbs to V-β1 or V-β2, which allowed both the stimulation and recognition of $CD3^+$ T cells expressing specific T cell receptors. PBL from healthy donors were exposed to anti-V-β1 or anti-V-β2 mAbs either in the presence of control beads or beads conjugated with anti-CD3/CD28/CD40 mAbs.

The presence of anti-CD3/CD28/CD40 beads completely inhibited the induction of an immune response specific for anti-V-β1 or anti-V-β2 (data not shown). In contrast, as shown in two representative experiments (Table 4), signaling via these beads expanded the proliferation of V-β-specific T lymphocytes that had been previously activated. Lymphocytes primed by exposure to anti-V-β1 or anti-V-β2 proliferated, with retained specificity for the given anti-V-β, in response to the respective anti-V-β alone or in combination with anti-CD3/CD28/CD40 beads. Similarly, V-β specificity was retained when cells activated by anti-V-β were expanded with the anti-CD3/CD28/CD40 beads in the presence of the same anti-V-β mAb, a different one, or no such mAb. Stimulation via anti-V-β mAb together with activation signals via anti-CD3/CD28/CD40 beads led to higher production of TNF than achieved by antigen-specific stimulation or activation alone (Table 4, experiment 1). The presence of anti-CD3/CD28/CD40 beads protected against the inhibitory effect of TGF-β1 on lymphocyte proliferation and allowed the production of significant amounts of IFN-γ 2 days after removal of the beads from the lymphocytes and with TGF-β1 remaining in the culture medium (Table 4, experiment 2). With the anti-CD3/CD28/CD40 beads, >20,000 pg IFN-γ was detected in the culture medium whether or not TGF-β1 was present (data not shown). The number of T cells

Table 4. Secondary sensitization of PBL from two healthy donors in the presence of beads conjugated with anti-CD3/CD28/CD40 mAbs (or unconjugated beads, as controls) using anti-V-β1 or anti-V-β2 as surrogate antigens

Exp.	1st stimul. on anti-V-β	2nd stimul. on anti-V-β	Anti-CD3/CD28/CD40 beads	TGF-β	Lymphocytes (× 10^6)			Lymphokine pg/ml
					Total	V-β1	V-β2	
1	1	1	−	−	12.1	11.4	0.04	150
	1	2	−	−	2.5	1.9	0.05	NT
	1	None	−	−	1	0.79	0.01	1
	1	1	+	−	11.9	9.3	0.12	1,760
	1	2	+	−	14.0	11.3	0.28	NT
	1	None	+	−	15.7	12.9	0.16	880
	2	1	−	−	0.9	0.03	0.54	NT
	2	2	−	−	3.5	0.03	2.5	340
	2	None	−	−	0.5	0.005	0.32	2
	2	1	+	−	14.7	0.15	7.6	NT
	2	2	+	−	11.5	0.23	5.1	5,040
	2	None	+	−	13.9	0.14	7.2	1,960
2	1	1	−	−	12.1	8.0	0.1	1,220
	1	1	−	+	1.1	0.8	0.1	194
	1	1	+	−	14.9	6.5	0.1	1,546
	1	1	+	+	11.0	8.2	0.3	405
	1	2	−	−	4.4	3.7	0.3	47
	1	2	−	+	1.8	1.6	0.2	47
	1	2	+	−	19.1	12.1	0.2	536
	1	2	+	+	16.9	12.8	0.3	161

TGF-β1 (5 ng/ml) was added as indicated in exp. 2. Lymphocyte numbers (× 10^6) and lymphokine production (TNF in exp. 1 and IFN-γ in exp. 2) was measured 2 days after removal of the beads. NT, not tested.
*>90% of the lymphocytes are CD3-positive according to FACS analysis.

in groups exposed to anti-V-β1 and not receiving TGF-β1 was approximately the same whether or not anti-CD3/CD28/CD40 beads were present, whereas T cell proliferation sharply decreased when the group stimulated only via anti-V-β1 was exposed to TGF-β1. Consequently, the observed protection of CD3 engagement against inhibition by TGF-β1 was not an artifact caused by fewer molecules of TGF-β1 per T lymphocyte.

Discussion

Although initial experiments showed that PBL and TIL from patients with stage IV cancer secreted TNF and IFN-γ when cocultivated with autologous tumor cells, the lymphokine levels were extremely low (particularly in cultures lacking monocytes), and there was no lymphocyte proliferation. In contrast, lymphokine production was dramatically increased, and there was vigorous lymphocyte proliferation when we used a procedure (19, 20) in which beads conjugated with a mAb to CD3 in combination with mAbs to CD28 and/or CD40 were added to the cultures. Tumor-selective CTL, which were MHC class I-restricted and $CD8^+$, could be generated from lymphocytes that had been activated over 4–5 days by anti-CD3/CD28 or anti-CD3/CD28/CD40 beads in the presence of autologous tumor cells (and monocytes) and then expanded in the absence of tumor cells and beads. We conclude that stimulation of T lymphocytes via CD3 (and costimulatory signals) expands all T lymphocytes and facilitates the generation of CTL in the presence of autologous tumor cells and antigen-presenting cells in the cultures. The low proliferation and lymphokine production of unstimulated PBL harvested from cancer patients correlated with their low expression of CD3, CD28, CD4, and CD8. Exposure to anti-CD3/CD28 or anti-CD3/CD28/CD40 beads up-regulated lymphocyte expression of CD3 and CD28, as it increased their ability to proliferate and form lymphokines. Increased expression of CD3 was not the result of a preferential expansion of lymphocytes that originally had high density of CD3 expression.

Our data indicate that at least some patients with advanced cancers have T lymphocytes that can mount tumor-destructive immune reactions but are inhibited from doing so *in vivo*, and that signals mediated via CD3 in the presence of tumor antigens can activate these reactions. Because the view that polyclonal stimulation via CD3 can activate antitumor immunity challenges the current concept that such stimulation prevents or overrides recognition of antigen by the T cell receptor, experiments were performed in a model, using mAbs to V-β1 or V-β2 as surrogate antigens. These experiments demonstrated that signals mediated via CD3 dramatically expanded the proliferation of already primed T lymphocytes without loss of their V-β specificity whereas they prevented the *de novo* induction of a specific response. Furthermore, exposure of T lymphocytes to the specific anti-V-β together with anti-CD3/CD28/CD40 beads was optimal in inducing the production of TNF.

Tumor cells exposed to lymphocytes and stimulated via CD3 in combination with CD28 and/or CD40 were regularly destroyed within 24–48 h by a mechanism that had no detectable antigen specificity, although TNF production appeared to be greater in the presence of autologous tumor cells than allogeneic tumor cells or fibroblasts. Most likely, polyclonal stimulation of CD3 plus costimulation via CD28 and CD40 produced lymphokines that activated natural killer cells and monocytes and also (like TNF) had a direct toxic effect. We hypothesize that lymphocyte activation, accompanied by tumor cell killing, causes the release of antigen, which is taken up and processed by monocytes in the cultures that differentiate into dendritic cells and present epitopes for the selective expansion of tumor-reactive T cells. Therapeutic vaccines may be based on the same principle to activate and expand suppressed lymphocytes in tumor-bearing individuals and also may facilitate the generation of immune responses to subdominant epitopes. It is noteworthy that treatment of tumor-bearing mice with anti-CD3 mAb has been shown to have antitumor activity under certain circumstances (26).

The procedures we have used make possible the generation of $CD83^+$ dendritic cells and $CD3^+$ lymphocytes, which continue to expand over >10 weeks of *in vitro* culturing and should lend themselves to adoptive immunotherapy. This finding may be because costimulation via CD28 decreases the probability for lymphocytes to undergo apoptosis (27, 28), providing them with a long lifespan *in vitro* (19). Costimulated lymphocytes also have survived for a long time after transfer back to autologous patients (29) as opposed to lymphocytes expanded in the presence of high doses of IL-2.

Most of the patients died within a year of donating PBL or TIL, despite our evidence that their T cell repertoire at least in some cases included lymphocyte clones that could recognize the tumors as antigenically foreign. Most likely, the failure of these clones to expand *in vivo* and differentiate into effector cells was due to the production by the tumor and/or the host of molecules that down-regulated or terminated T cell reactivity. Many such molecules are known (9) with members of the TGF-β1 family being among the most powerful. The effects of TGF-β1 were thus investigated. It is encouraging that T cell stimulation via CD3 in combination with CD28 alone or together with CD40 protected against ≈50% of its inhibitory effect on lymphocyte proliferation and production of TNF and IFN-γ, even when the TGF-β1 was used at saturation levels of 20 ng/ml in the cultures. Likewise, T cell stimulation via anti-CD3/CD28/CD40 beads protected against the inhibitory effect of TGF-β1 in the anti-V-β model.

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Clinical-Scale Selection of Anti-CD3/CD28–Activated T Cells After Transduction with a Retroviral Vector Expressing Herpes Simplex Virus Thymidine Kinase and Truncated Nerve Growth Factor Receptor

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ABSTRACT

Activation of T cells is necessary for efficient retroviral-mediated gene transfer. In addition, if the population of infused cells is to be limited to transduced cells, a means of positive selection is required. We describe a clinical scale procedure for activation of donor T cells with anti-CD3/CD28 beads followed by transduction with a retroviral construct expressing the herpes simplex virus thymidine kinase (HSV-tk) and human nerve growth factor receptor (NGFR). Optimization of transduction parameters was performed, testing the timing of transduction, centrifugation, and the use of serum. In large-scale experiments, 3–5 × 10^8 peripheral blo d mononuclear cells (PBMC) were activated with anti-CD3/CD28 beads and expanded to day 13. Transduction was accomplished using MFG-TKiNG supernatant produced from the PG13 packaging line 48 hr after T-cell activation. The mean transduction frequency was 37.5% based on NGFR expression, and the mean expansion observed was 42.6-fold (mean final cell number 1.85 × 10^{10}). A comparison of the ability of the Baxter Isolex 300i and the Miltenyi CliniMACS to perform purification of NGFR+ cells suggests that greater purity can be achieved with the CliniMACS device (67.4% vs. 97.7%), while the yield of transduced cells appears higher with the Isolex 300i (41.3% vs. 23.5%). We conclude that a strategy based on activation of human T cells with anti-CD3/CD28 beads can result in sufficient transduction, expansion, and purification based on NGFR expression for clinical trials.

OVERVIEW SUMMARY

The potential use of T cells engineered with negative selectable elements such as the herpes simplex virus thymidine kinase gene may prove advantageous by achieving additional efficacy and safety associated with allogeneic transplantation. If retroviral-mediated gene transfer is to be used, the development of clinical scale methodology for the activation, transduction, expansion, and selection of sufficient T cells is of great importance, and is the focus of these investigations. Because of concerns regarding the possible induction of apoptosis via activation through the T-cell receptor alone and/or interleukin-2, we chose to test magnetic beads with anti-CD3 as well as anti-CD28 antibodies to obtain activation to allow efficient transduction. The roles of retroviral packaging lines, centrifugation, and serum were evaluated. In addition, the utilization of the nerve growth factor cell surface antigen and magnetic bead technology as a positive selection strategy was tested to compare magnetic bead devices to determine the relative purity and yield that can be achieved in preclinical studies.

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is an important therapeutic modality for malignancies, marrow failure, metabolic disorders, and immunodeficiencies. Unfortunately, the morbidity and mortality associated with graft versus host disease (GVHD) remains high (Nademanee *et al.*, 1995; Davies *et al.*, 1996). The cells primarily responsible for GVHD are donor T cells, and the incidence and severity of GVHD can be decreased with T-cell depletion (TCD) of the graft. However, the removal of the T-cell population may be associated with an increased risk of graft failure, and may also adversely affect immune reconstitution (Martin *et al.*, 1985; Marmont *et al.*, 1991; Davison *et al.*, 2000). In addition, the beneficial immunologic surveillance provided by donor T cells decreases the relapse rate of recipients undergoing transplantation for leukemia, and this graft versus leukemia (GVL) effect may be diminished by T-cell depletion (Gale and Horowitz, 1990; Horowitz *et al.*, 1990; Enright *et al.*, 1996). Therefore, strategies that allow the administration of donor T cells to facilitate engraftment and GVL while providing an additional means of controlling GVHD may prove beneficial.

One approach developed to address these issues is the genetic engineering of donor T cells with a gene providing the potential for negative selection (Bonini *et al.*, 1997; Tiberghien *et al.*, 1997). This "suicide gene" approach allows the eradication of the engineered cell population with a prodrug that is relatively nontoxic to cells not expressing the gene. Thus, this approach could allow the inclusion of T cells with their positive effects on engraftment, GVL, and immunologic function while providing an additional measure of protection from severe GVHD. There have been reports suggesting that this is feasible in murine models (Cohen *et al.*, 1997; Contassot *et al.*, 2000), and may prove advantageousin a clinical setting (Bonini *et al.*, 1997; Tiberghien, 2001; Tiberghien *et al.*, 2001). The successful implementation of this procedure requires the resolution of several obstacles. If retrovirus-mediated gene transfer is used, the target cells must be proliferating for effective proviral integration (Miller *et al.*, 1990). Second, because it is unlikely that all T cells will be transduced, some means of positive selection will be required to ensure that the T cells to be infused contain the capacity to provide negative selection. In addition, the T cells that have undergone activation, transduction, and selection may prove phenotypically or immunologically distinct from unmanipulated T cells ordinarily infused with an marrow allogeneic graft. Finally, the ability to eliminate the transduced cell population at a concentration of prodrug that can be achieved in the serum of patients undergoing HCT is necessary.

We report the results of our clinical-scale cell production, transduction, and selection studies using magnetic beads with antibodies to both CD3 and CD28 to provide the activation signals to T cells to achieve high-efficiency transduction. Signaling through both the T-cell receptor and the costimulatory molecule CD28 has been documented to initiate proliferation of T cells (Levine *et al.*, 1998; Garlie *et al.*, 1999; Shibuya *et al.*, 2000), and may decrease the development of anergy or apoptosis associated with stimulation through the T-cell receptor alone (Wolf *et al.*, 1994; Noel *et al.*, 1996; Muller *et al.*, 1999). The use of anti-CD3 antibodies and interleukin-2 (IL-2) has been shown to generate large numbers of T cells (Anderson *et al.*, 1988; Ochoa *et al.*, 1989), and has been used to provide transduced T cells for clinical use (Tiberghien *et al.*, 2001). However, there is concern that the use of antibodies capable of cross-linking the T-cell receptor alone may result in eventual apoptosis of the activated cells, which may be prevented with stimulation via the CD28 costimulatory pathway, possibly by modifying expression of bcl-2 and bcl-x (Noel *et al.*, 1996; Radvanyi *et al.*, 1996; Muller *et al.*, 1999). We therefore chose to test the potential to achieve transduction of human T cells utilizing anti-CD3/CD28 antibodies bound to magnetic beads (Levine *et al.*, 1995, 1996). We have utilized a vector expressing the herpes simplex virus thymidine kinase (HSV-tk) gene to provide the capacity for negative selection. Transduced cells were identified and purified on the basis of the cell surface expression of the truncated human nerve growth factor receptor (NGFR). The Baxter Isolex 300i (Nexell Therapeutics Inc., Irvine, CA) and Miltenyi CliniMACS (AmCell Corp., Burlingame, CA) clinical cell separation devices were compared to determine purity and yield of NGFR+ human T cells. We determined that peripheral blood mononuclear cells (PBMC) activated with anti-CD3/CD28 beads and IL-2 achieved a sustained proliferative response. In association with a transduction protocol using a vector packaged to make use of gibbon ape leukemia virus (GALV) envelope and centrifugation, transduction frequencies of 40%–60% were achievable. In large-scale transduction experiments, the Baxter Isolex 300i appeared to provide a greater yield of NGFR+ cells, while a higher degree of purity was achieved with the Miltenyi CliniMACS device. We believe this is the first report of a clinical-scale protocol in which anti-CD3/CD28 beads are used to achieve T-cell transduction and selection on the basis of expression of a cell surface antigen such as NGFR.

MATERIALS AND METHODS

Mammalian cell culture

NIH 3T3 cells, PA317 and PG13 cells were maintained in Dulbeccos's Modified Essential Medium (DMEM) supplemented with 10% newborn calf serum (Sigma, St. Louis, MO) and penicillin/streptomycin (GIBCO BRL, Rockville, MD). Retroviral supernatants were generated in small scale from confluent retroviral producing lines in 75-cm^2 flasks (Corning, Corning, NY) at 32°C over 16–24 hr. Large-scale virus production was performed using a NUNC cell factory apparatus (Cat. #164327; NUNC, Naperville, IL) by placing fresh medium every 24 hr on the confluent producer line maintained at 32°C as long as the cells remained adherent. Supernatants generated from viral producer lines were filtered using 0.22-μm filters (Cat. #357111, Becton Dickinson Labware, Franklin Lakes, NJ) to remove any residual producer cells. In proliferation assays 10^5 cells were placed in a total of 200 μl of medium in 96-well plates, and were pulsed for 16 hr with 1 μCu of [^{3}H]thymidine (Perkin Elmer Life Sciences Inc., Boston, MA) prior to cell harvesting and determinations of thymidine incorporation (cell harvester, matrix 9600 reader; Packard Instruments, Downer's Grove, IL).

Blood samples were obtained from volunteer donors in accordance with University of Minnesota Institutional Review Board (IRB)-approved protocols using heparin-containing 10-ml

tubes; in situations in which a unit of whole blood was used, 5000 units of heparin were added to the bag containing the specimen prior to processing. PBMC were obtained using histopaque (Cat. #1077-1, Sigma) separation. The cells were maintained in X-VIVO 15 (Biowhittaker; Walkersville, MD) with penicillin/streptomycin and either 10% fetal bovine serum (FBS) or human frozen plasma (FP) purchased from the Red Cross (St. Paul, MN). Prior to addition to X-VIVO 15, the FP was thawed, maintained at 55°–57°C for 1 hr and centrifuged at 3000 rpm (2510*g*) for 10 min at room temperature. Using a plasma extractor, the plasma was expressed into 300-ml transfer bags, allowing any clots to remain in the original bag. Activation of PBMC was achieved with anti-CD3/CD28 beads added to the cells in culture on day 0 at a 3:1 ratio to the number of T cells (Levine *et al.*, 1998; Garlie *et al.*, 1999). IL-2 (Chiron Corp., St. Louis, MO) was used in individual experiments as described.

Monoclonal antibodies and flow cytometric analysis

The anti-NGFR hybridoma 20.4 (murine IgG1) was obtained from ATCC (HB 8737, 200-3-G6-4; clone 20.4) and antibody produced for our laboratory by Taconic BioServices (Germantown, NY). Depending on the experiment, either unconjugated or biotinylated antibody was used for detection or selection of NGFR + cells. Additional antibodies used for phenotypic analysis including anti-CD3 were obtained from Becton Dickinson; flow cytometric analysis was performed using a Becton Dickinson FACSCaliber.

Construction of the MFG-TKiNG vector

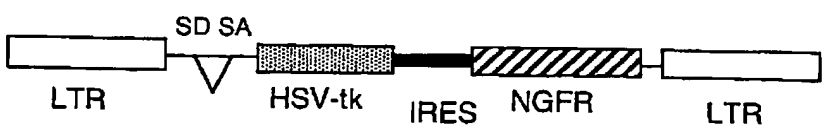
The MFG-TKiNG retroviral construct was designed to express a truncated form of NGFR and HSV-tk using the MFG strategy, which has been shown to provide high-efficiency gene transfer and expression (Dranoff *et al.*, 1993; Jaffee *et al.*, 1993). The GCsamE75t vector consists of the Moloney murine leukemia virus long terminal repeats (LTR), retroviral splice donor/acceptance sites, a polylinker including the *Not*I and *Xho*I sites, an internal ribosomal entry site (IRES) and the NGFR cDNA. The HSV-tk gene was isolated from plasmid pHSV-106 (Bethesda Research Laboratories Inc., Gaithersburg, MD) and was ligated into the *Not*I/*Xho*I polylinker in frame with the NGFR gene using the encephalomyocarditis IRES (Zitvogel *et al.*, 1994; Gallardo *et al.*, 1997) to express both the NGFR and HSV-tk genes from a single bicistronic message, optimizing the potential that the HSV-tk gene will be expressed in cells selected on the basis of NGFR (Fig. 1). The construct was cotransfected into the PA317 amphotropic line (Miller and Buttimore, 1986) with the pFR400 plasmid containing the Arg22 dihydrofolate reductase (DHFR) gene and colonies selected in 1.0 μM methotrexate (Simonsen and Levinson, 1983). Clones were screened for their production of HAT resistant colonies on NIH 3T3 tk^- cells as previously described (Vinh and McIvor, 1993). The highest titer observed was 0.8×10^6 colony-forming units per milliliter (cfu/ml) of supernatant. Supernatants from PA317 clones were used to transduce the PG13 line (Bauer *et al.*, 1995; Bunnell *et al.*, 1995), and clones selected on the basis of NIH 3T3 tk^- expression using flow cytometry to sort the NIH 3T3 tk^- population with the anti-NGFR antibody 20.4. Because the GALV receptor is not expressed on murine cells, the titer of these clones could not be determined on NIH 3T3 tk^- cells; therefore, the relative rates of transduction were determined in donor-derived human T cells.

T-cell transduction assay

In the small-scale analysis used to optimize transduction conditions, transduction of human T cells was performed 48 hr after activation of T cells, unless otherwise noted. Ficoll separation was used to obtain PBMC, which were activated with anti-CD3/CD28 beads at a ratio of 3 beads per cell in 1000 units of IL-2 per milliliter in X-VIVO 15 medium with 10% FBS, unless otherwise stated. After 48 hr, 2×10^5 cells were placed in 15-ml polypropylene tubes (Corning) in 0.4 ml of X-VIVO 15 and 1.2 ml of retroviral supernatant; protamine (American Pharmaceutical Partners, Inc., Los Angeles, CA) was added to achieve a final concentration of 8 μg/ml. Unless otherwise indicated, the cells were centrifuged at 4000*g* at 32°C for 1 hr in a JS-30 rotor in a J-6B centrifuge (Beckman Coulter, Inc., Fullerton, CA), and incubated for 16 hr at 37°C in 5% CO_2. The cells were washed and placed in fresh culture medium, and after 48 hours, flow cytometry was performed with 100 ng of the unconjugated 20.4 anti-NGFR antibody and 10 μl of a secondary anti-murine immunoglobulin G (IgG) antibody conjugated to phycoerythrin (PE) (Sigma). The cells were also stained with an anti-CD3 antibody conjugated to fluorescein isothiocyanate (FITC). In other experiments, modifications in the transduction procedure were made as noted in the figures.

Clinical-scale experiments

To obtain sufficient cells for large-scale testing, 1 unit of whole blood was procured from volunteer donors in accordance with an IRB-approved protocol, and Ficoll separation performed. On day 0 a total of $3–5 \times 10^8$ PBMC were activated with anti-CD3/CD28 beads at a 3:1 ratio, and the cells were cultured in X-VIVO supplemented with 10% FP and penicillin/streptomycin to achieve a final concentration of 0.5×10^6 cells/ml in 1000 units of IL-2 per milliliter in a 3-liter Lifecell bag (Dynal Biotech Inc., Lake Success, NY). On day 2, 1.5 liters of retroviral supernatant (PG13-derived) and protamine



MFG-TKiNG Virus

FIG. 1. MFG-TKiNG retrovirus. The herpes simplex virus thymidine kinase gene (HSV-tk) and the truncated nerve growth factor receptor (NGFR) are coexpressed using the internal ribosome entry site (IRES) from the encephalomyocarditis virus. Splice donor (SD) and splice acceptor (SA) sites are designated.

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(final concentration, 8 μg/ml) were added to the cells. The product was separated into six 600-ml transfer bags (Terumo Medical Corporation, Somerset, NJ) and centrifuged at 3710 rpm (4000g) for 1 hr at 32°C in a Sorvall RC-3B centrifuge and then carefully removed and placed upright in a 37°C 5% CO_2 incubator. On day 3 the cells were washed and placed in fresh medium in a concentration of 0.5×10^6 cells/ml. Fresh medium was added every 2–3 days to keep the cell number less than 2×10^6 cells/ml until day 13, when the anti-CD3/CD28 beads were removed using the secondary magnet of the Baxter Isolex 300i device (Nexell Therapeutics Inc.). A sample of cells was obtained to determine the proportion expressing NGFR, and the remainder was subjected to separation of the NGFR+ fraction using the Isolex 300i or the CliniMACS device. To perform the isolations, 1.0 ml of the anti-NGFR monoclonal antibody 20.4 (2.4 mg) was added to the cells. In the case of the experiments using the Isolex 300i, an unconjugated 20.4 antibody preparation was used in association with the sheep anti-murine antibody/bead reagent currently being used in clinical trials according to manufacturers' specifications. For selections using the CliniMACS device, 1.0 ml of a biotinylated 20.4 antibody was used with streptavidin beads (Miltenyi Biotech Inc.) according to specifications, using the tubing sets and buffers designed for the CliniMACS.

RESULTS

Activation and timing of transduction in donor-derived T cells

To achieve high-efficiency retrovirus-mediated gene transfer it is critical to induce the target cell population to undergo mitosis to facilitate integration (Miller *et al.*, 1990; Stevenson *et al.*, 1990). In these studies, anti-CD3/CD28 beads were utilized to activate T cells by cross-linking the T-cell receptor and costimulatory pathways simultaneously. We first sought to determine the rate at which proliferation was achieved in PBMC after exposure to the anti-CD3/CD28 beads, comparing thymidine incorporation to cells induced with IL-2 alone or both IL-2 and beads. Active proliferation was observed in cells pulsed 24-hr postactivation with the beads, and was not affected by the presence or absence of exogenous IL-2 (100 U/ml in this experiment). Thymidine incorporation continued over the 96 hr of the assay in cells in the presence of anti-CD3/CD28 beads, whereas little proliferation was documented in the absence of the beads, despite the addition of IL-2 to the culture (Fig. 2).

Centrifugation as a means of enhancing T-cell transduction

The role of centrifugation as a strategy for increasing the efficacy of retroviral mediated gene transfer was tested to determine effects on transduction frequency and overall viability. PBMC activated with anti-CD3/CD28 beads were transduced 48 hr after activation, using protamine (8 μg/ml). In this experiment, two donors were used, and testing at each point performed in triplicate for each donor. The reported data is pooled from both donors ($n = 6$), as a similar trend was observed in both donors (Fig. 3). We observed a continuous increase in transduction frequency to 4000g with minimal effect in the viability of the transduced cells. We chose 4000g for subsequent experiments and did not continue to increase the g force, because there were concerns regarding the integrity of the bags with additional centrifugation.

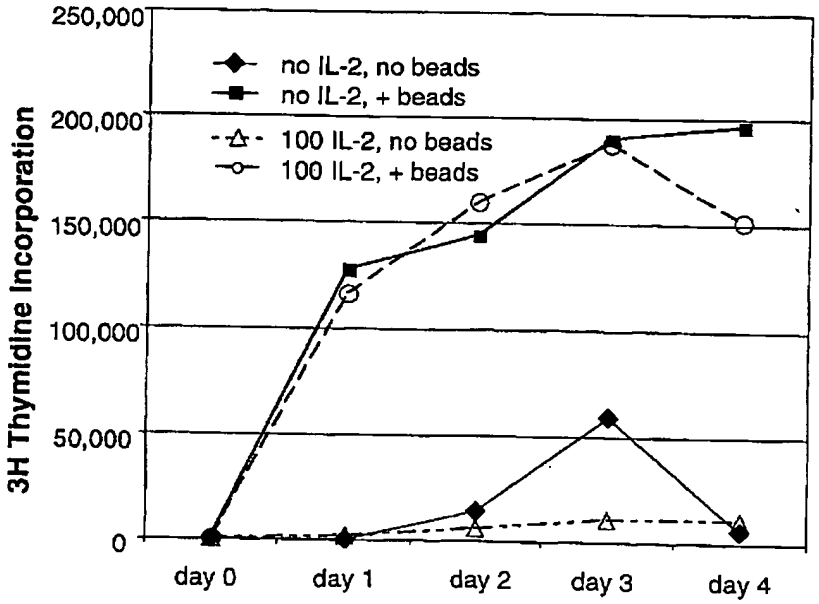

FIG. 2. Proliferation of human T cells with anti-CD3/CD28 beads. Donor-derived peripheral blood mononuclear cells (PBMC) were activated with anti-CD3/CD28 beads at a ratio of 3 beads per cell in the presence or absence of 100 units of IL-2 per milliliter. The day that PBMC were isolated, cells in each group were pulsed with [^{3}H]thymidine (day 0). Cells in identical groups were pulsed at 24, 48, 72 and 96 hr in culture, corresponding to day 1 through 4.

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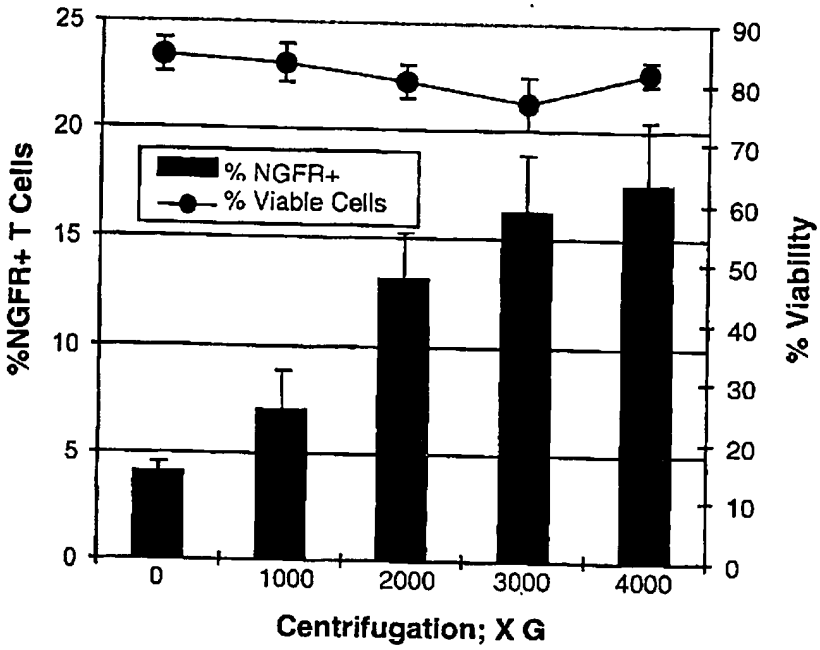

FIG. 3. Effect of centrifugation on transduction frequency and T cell viability. Peripheral blood mononuclear cells (PBMC) activated with anti-CD3/CD28 beads were transduced 48 hr postactivation in triplicate in X-VIVO 15 medium with 1000 U/ml IL-2. In each tube 2×10^5 cells in 400 μl of medium were transduced using 1.2 ml of MFG-TKiNG supernatant in 8 μg/ml protamine using centrifugation (0–4000*g*) for 1 hour at 32°C. Supernatant derived from PG13 supernatant was used for transduction. Trypan blue was used to determine viability while anti-nerve growth frequency receptor (NGFR) antibody was used to evaluate the frequency of transduction.

Transduction efficacy using PA317 and PG13 packaged vector

To achieve maximal transduction efficacy, we sought to determine if packaging lines containing the amphotropic murine leukemia virus (A-MuLV) or GALV envelope gene was most advantageous in achieving gene transfer and expression in anti-CD3/CD28 activated T cells. The MFG-TKiNG plasmid was initially transfected into the A-MuLV line PA317, and supernatant used to transduce PG13 cells, as described above. Donor-derived PBMC were exposed to retroviral supernatants 48 hr after activation in the presence of IL-2 (1000 U/ml) in triplicate. The advantage of using supernatant generated from the GALV line (PG13) was straightforward and highly significant (Fig. 4).

Timing of transduction after activation

The next goal was to establish the optimal timing of transduction after activation with the anti-CD3/CD28 beads; PBMC were activated and transduced in replicates of 3 using MFG-TKiNG supernatant 24, 48 or 72 hr after activation. In this experiment, 1.2 ml of retroviral supernatant (PG13-derived) was used to transduce 2×10^5 activated cells in 0.4 ml of medium; the proportion of cells expressing NGFR was determined by flow cytometry after transduction. This documents that efficient transduction (median, 58%) can be achieved with a single exposure of retroviral supernatant when transduction is performed 48 hr after initial activation (Fig. 5).

Role of serum products in the transduction of human T cells activated with anti-CD3/CD28 beads

The use of serum-free medium such as X-VIVO 15 may be advantageous in supporting the proliferation of T cells without a requirement for human or bovine serum. We therefore determined the effect of serum products such as FBS or FP on the transduction of T cells. From the initial placement of PBMC in X-VIVO 15 medium, no serum, 10% FBS, or 10% FP was provided within the medium, which continued throughout the culture period including the transduction procedure. Retroviral supernatant used for transduction in each group was from a single batch of supernatant produced in DMEM medium and 10% FBS. We observed that the addition of FBS to the medium increases transduction efficiency when compared to cells maintained in medium without serum, while the use of FP provided a significantly increased transduction frequency when compared to FBS (Fig. 6).

Large-scale expansion and selection of transduced T cells based on NGFR expression

For these experiments, volunteer donors provided 1 unit of whole blood from which PBMC were obtained. The mean number of PBMC was 5.8×10^8 cells (±2.4 standard deviations [SD]). For each experiment 5×10^8 PBMC were activated with anti-CD3/CD28 beads, except in two cases when less than 5×10^8 cells were obtained (2.9 and 3.0×10^8 cells) and expanded in the presence of anti-CD3/CD28 beads and IL-2 to day 13.

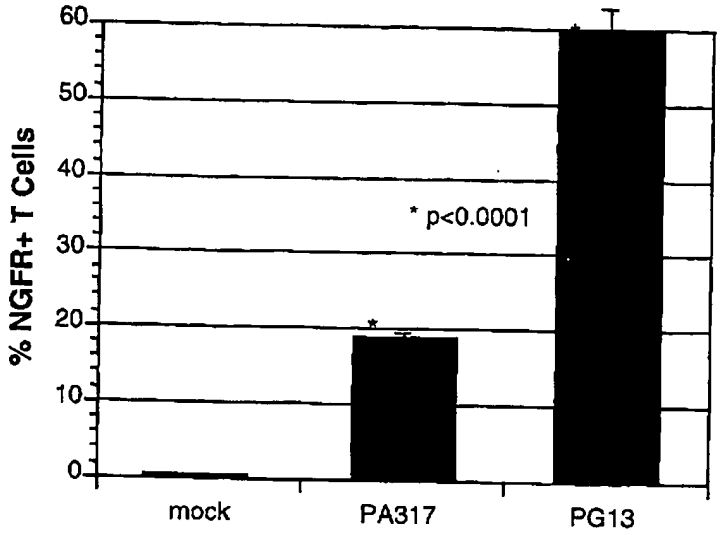

FIG. 4. Transduction of peripheral blood mononuclear cells (PBMC) with PA317 and PG13 supernatant. MFG-TKiNG supernatant derived from the PA317 and PG13 lines were used to transduce donor-derived T cells in triplicate 48 hr after activation by anti-CD3/CD28 beads in X-VIVO 15 medium with 1000 U/ml of IL-2 using protamine (8 μg/ml) and centrifugation (4000g for 1 hr at 32°C). Transduction frequency was determined by flow cytometric analysis of nerve growth factor receptor (NGFR) expression 72 hr posttransduction.

The mean number of viable cells present after activation, transduction, and expansion was 1.85×10^{10} ($\pm 0.93 \times 10^{10}$) cells, with a mean expansion of 42.6-fold. The cells were transduced with the MFG-TKiNG virus on day 2, and the mean transduction frequency was 37.5% ± 26.8% ($n = 5$). Because the purpose of this strategy is to ensure that infused T cells can be eradicated *in vivo* if severe GVHD is observed, a means of achieving purity of successfully transduced T cells on the basis of NGFR+ is of great importance. We therefore sought to compare the two clinical scale devices available for selection of cell surface antigens using magnetic beads. The Baxter Isolex 300i and the Miltenyi CliniMACS device have both been de-

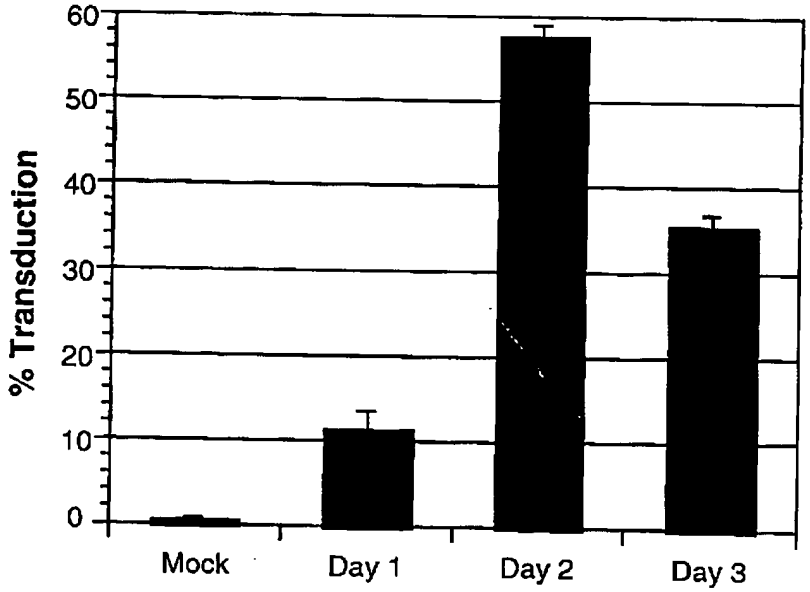

FIG. 5. Transduction frequency based on timing after activation. Donor-derived peripheral blood mononuclear cells (PBMC) were activated with anti-CD3/CD28 beads in X-VIVO 15 medium with 1000 U/ml IL-2, and 24 hr later 2×10^5 cells in 400 μl were transduced using MFG-TKiNG supernatant (1.2 ml) in the presence of 8 μg/ml of protamine (day 1). Transduction was performed using centrifugation (4000g for 1 hr at 32°C) and transduction allowed to proceed for 16 hr at 37°C. On day 2 (48-hr postactivation) the day 1 transduced population was washed and resuspended in fresh medium, while additional cells (day 2) underwent transduction using an identical procedure; similarly, transduction was performed in the final group (day 3) 72 hr after initial activation. The proportion of cells expressing nerve growth factor receptor (NGFR) was determined 48 hr after transduction of the third group.

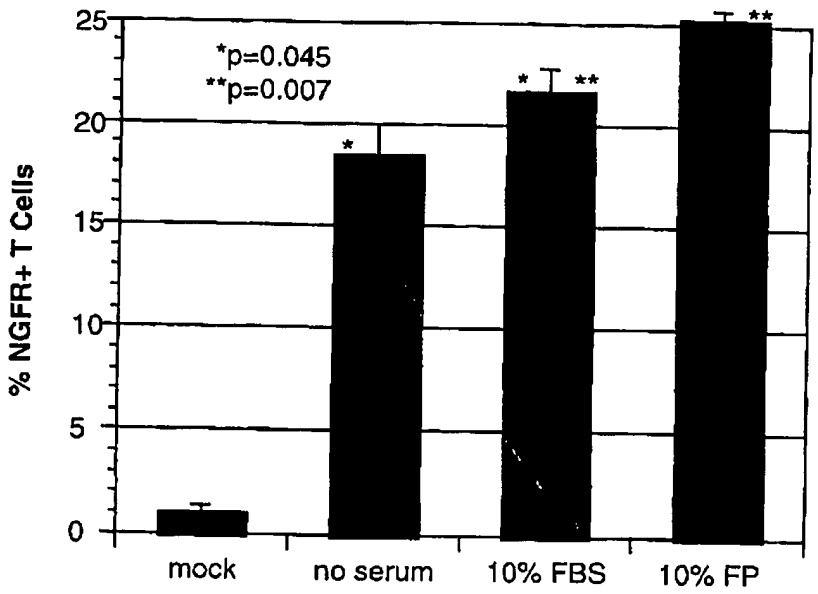

FIG. 6. Effect of serum and frozen plasma (FP) on transduction frequency. Peripheral blood mononuclear cells (PBMC) activated as described previously were cultured in X-VIVO 15 medium with 1000 U/ml of IL-2 alone (no serum) or with 10% fetal bovine serum (FBS) or human FP throughout the experiment. Transduction was performed 48 hr after activation using centrifugation and protamine as previously described.

veloped to perform positive selection of hematopoietic cells for clinical applications. In these experiments, the cells were divided after transduction and expansion and separations performed on both devices as described above. The findings suggest that greater purity may be obtained with the CliniMACS device (mean proportion of NGFR+ cells 97.7% vs. 67.4%, respectively; $p = 0.051$), while a greater recovery of NGFR+ cells was observed with the Isolex 300i (mean yield 41.3% vs. 23.5%), although statistical significance was not achieved (Fig. 7).

DISCUSSION

In this report we describe a preclinical protocol to generate large numbers of transduced human T cells after activation with anti-CD3/CD28 beads, using a retrovirus designed to express HSV-tk. A high degree of purity of the transduced cell population is achieved on the basis of NGFR using magnetic beads and clinical scale devices. To develop procedures designed to generate sufficient numbers of cells for clinical trials, activation of T cells is of importance to achieve transduction. We chose to investigate the use of anti-CD3/CD28 beads to activate T cells in experiments designed to test the feasibility of this approach to provide a protocol sufficient to generate cells for clinical purposes. The use of this technique has been reported as resulting in a population of T cells that can be continuously stimulated to actively proliferate (Levine *et al.*, 1996; Garlie *et al.*, 1999). Our data confirm that highly efficient gene transfer can be achieved in human T cells with a single exposure to the MFG-TKiNG retrovirus. These findings are similar to those of other investigators using immobilized anti-CD3 and anti-CD28 antibodies (Pollok *et al.*, 1999; Koehne *et al.*, 2000). In addition, Quinn et al. described the potential to transduce human T cells after activation with these antibodies immobilized on beads (Quinn *et al.*, 1998), although not in numbers sufficient for clinical use and without the capacity to perform positive selection on the basis of the expression of cell surface antigens. The described protocol provides efficient retroviral-mediated gene transfer, expansion, and selection using clinical scale magnetic bead devices.

The choice of packaging cell line has important implications for the transduction of human hematopoietic cells. The PA317 line, expressing the amphotropic murine leukemia virus (A-MuLV) envelope, has been well-tested clinically and provides the advantage of facilitating titering of virus using murine thymidine kinase negative (tk-) cell lines (Tabin *et al.*, 1982; Miller *et al.*, 1985). In contrast, the PG13 line was established to express the GALV envelope, and supernatants produced using this line have been shown to provide enhanced gene transfer in human hematopoietic cells (Bauer *et al.*, 1995; Bunnell *et al.*, 1995). However, because virus produced using PG13 cells is unable to transduce murine cells, the use of other means to quantitate the MFG-TKiNG virus is necessary. Comparison of the PA317 derived supernatant (titer of 0.8×10^6 cfu/ml based on determinations using the tk-3T3 cells) and PG13 supernatants in donor-derived T cells documents the increase in transduction frequency achieved with the latter.

Centrifugation has been used to enhance the physical association of retrovirus and target cells, and the effects on transduction have been shown to increase with the *g* force utilized as well as the duration of centrifugation (Bahnson *et al.*, 1995). We were interested in determining the effect of centrifugation on transduction frequency and T-cell viability. An additional issue in large-scale transduction procedures in the capacity for tissue culture bags to withstand the centrifugation procedure. Our data support previous observations that increases in the *g* forces associated with centrifugation increases the transduction frequency, while little decrease in viability was observed to 4000*g*. The 600-ml transfer bags were not susceptible to failure as tested.

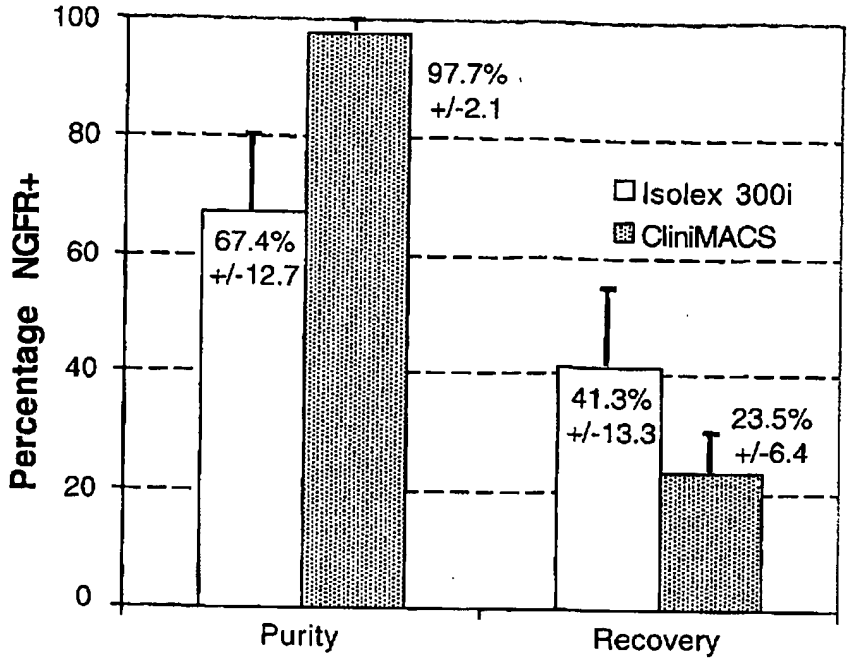

FIG. 7. Comparison of selection of nerve growth factor receptor-positive (NGFR+) cells using the CliniMACS and Isolex 300i Devices. Large-scale transduction experiments were performed using a mononuclear cell population derived from a unit of whole blood after separation using Ficoll. The cells were activated with anti-CD3/CD28 beads and transduced 48 hr later in X-VIVO 15 with 10% frozen plasma (FP) and 1000 U/ml of IL-2. Expansion continued until day 13, at which time the cells were divided and selected on the basis of NGFR expression using the Baxter Isolex 300i or Miltenyi CliniMACS device. The expression of NGFR was determined by flow cytometric analysis prior to and after selection. The total yield was determined by calculating the number of NGFR+ cells in a given cell fraction, and comparing this to the number of cells and the proportion that were NGFR+ after selection.

Modification of T cells for the purposes of both purification of transduced cells (positive selection) as well as the ability to eradicate the cells *in vivo* (negative selection) requires expression of two genes from the same virus. The retrovirus constructed for this purpose (MFG-TKiNG) has been designed to express the truncated NGFR gene as well as HSV-tk using the MFG strategy, which has been shown to provide efficient gene transfer and expression in a number of target cells (Dranoff *et al.*, 1993; Jaffee *et al.*, 1993). Our laboratory has previously used MFG virus successfully in human natural killer (NK) cells (Miller *et al.*, 1997). The positive selectable element utilized in the MFG-TKiNG viral construct is the NGFR gene, which has been shown to be expressed well on human T cells (Mavilio *et al.*, 1994; Valtieri *et al.*, 1994). The NGFR molecule also has the potential advantage of being less immunogenic, because it has been documented that immunologic responses can be directed against antibiotic resistance genes (Riddell *et al.*, 1996; Verzeletti *et al.*, 1998). The use of the IRES in the vector design was designed to provide an additional measure of safety, providing expression of both genes on the same bicistronic message while selecting on the basis of the second gene. We observed significant variation in the rates of transduction in donor derived human T cells from experiment to experiment, likely caused by the titer of supernatant as well as differences between donors and experimental variation.

Culture conditions may prove of significant importance in optimizing the expansion, viability, and function of transduced T cells. We sought to test the importance of FBS and human FP in achieving efficient transduction of activated T cells, and documented that FP is a viable alternative to the use of bovine products during the transduction period. It appears that the processing of the plasma as described removes complement or other agents that have been described to interfere retroviral mediated gene transfer (Shimizu *et al.*, 1995). The role of IL-2 in the optimization of the expansion and subsequent function of transduced and selected T cells remains unclear. Current investigations are proceeding in our laboratory to determine if IL-2 can be limited or eliminated, and how this effects transduction, expansion, and the ability of T cells to respond to a second stimulus.

In summary, we describe a successful preclinical protocol in which activation of human T cells is achieved with anti-CD3/CD28 beads, facilitating efficient retroviral mediated gene transfer and selection of the transduced population using clinical scale magnetic beads devices.

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Use of anti-CD3/CD28 mAb coupled magnetic beads permitting subsequent phenotypic analysis of activated human T cells by indirect immunofluorescence

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Abstract

Functional analysis of T lymphocytes requires in vitro stimulation of these cells under experimental conditions that mimic as closely as possible physiological in vivo stimulation and that involve antigen/T cell receptor (TCR)-mediated activation. Because of the low frequency of antigen-specific T cells in human clinical samples, stimulation with a combination of anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) is a preferred method. Interaction of these mAbs with their ligand results in modulation of the mAb–ligand complex from the cell surface. However, as a result of incomplete modulation, CD3/CD28 mAb complexes often remain at the cell surface, thereby precluding subsequent indirect immunofluorescence and flow cytometry analysis using mouse immunoglobulin (Ig)-specific antibodies. This is of importance in situations in which no specific fluorochrome-conjugated mAbs are available, such as in screening procedures of Ig-containing hybridoma culture supernatants. We propose here the use of CD3/CD28 mAbs, linked to magnetic beads allowing standardization of the activation conditions, optimal activation of T cells and complete modulation of antigen–antibody complexes from the cell surface.
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Keywords: T lymphocytes; Cellular activation; Monoclonal antibodies; Magnetic beads; Immunofluorescence; FACS; Cytokine production

1. Introduction

Optimal activation of human T cells in vivo is ensured by the interaction of specific antigen with the T cell receptor (TCR) in the presence of co-stimulatory signals, provided for by antigen-presenting cells (APC). As the frequency of human antigen-specific T cells in peripheral blood mononuclear cell samples is very low and autologous or HLA-matched APC are rarely available, stimulation of T cells in vitro is often carried out with polyclonal activators such as the lectins PHA or Con A or with the combination of the phorbol ester TPA and calcium ionophore. Although these modes of activation may provide useful information on signalling pathways in T cells, they are, from a biological point of view,

Abbreviations: mAb, monoclonal antibody; Ig, immunoglobulin; TCR, T cell receptor; APC, antigen-presenting cells.

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artificial and not always comparable to in vivo stimulation conditions. For example, cytokine production profiles of human T cells, following stimulation with TPA and phorbol ester stimulation, differ from those that result from stimulation with specific antigen and APC (Yssel et al., 1992; Byun et al., 1994; Yang et al., 1995). A more physiologically relevant mode of activation is stimulation of T cells with a combination of a monoclonal antibody (mAb) specific for CD3, which mimics antigen-mediated T cell receptor (TCR)/CD3 complex-mediated signalling (Reinherz et al., 1982), and an anti-CD28 mAb which provides the required co-stimulatory signals (Hara et al., 1985; Geppert and Lipsky, 1987; Schwartz, 1990). Optimal stimulation of highly purified T cells requires the presence of accessory cells (Davis et al., 1986). However, this requirement is bypassed by the use of multivalent anti-CD3 mAb, either cross-linked onto tissue culture plates (Geppert and Lipsky, 1987), covalently linked to Sepharose beads, or in combination with an anti-mouse immunoglobulin (Ig)G (Umetsu et al., 1987), which results in cross-linking of CD3 on the cell surface. In contrast, triggering of CD28 does not require such cross-linking (Hara et al., 1985) and T cell activation is carried out with soluble mAbs (Levine et al., 1996, 1997).

Interaction of CD3 with a specific mAb results in its down-regulation and subsequent internalization of the CD3–mAb complex (Antel et al., 1982; Reinherz et al., 1982; Telerman et al., 1987). However, total loss of cell surface protein/mAb expression is dependent on optimal experimental conditions in vitro, as well as on the reagents used, and often, due to partial modulation, such complexes remain at the cell surface (see present study). The remainder of these protein/mAb complexes will interfere with subsequent immunostaining of cell surface molecules expressed on these cells, for which no directly conjugated mAbs are available.

In the present study, we have analyzed whether the use of mAbs linked to magnetic beads results in complete modulation of CD3/CD28 mAb complexes from the surface of T cells, thus permitting staining of other cell surface molecules expressed on these cells by indirect immunofluorescence and flow cytometry.

2. Materials and methods

2.1. Cells and culture conditions

Mononuclear cells were isolated from freshly collected peripheral blood from healthy individuals (Etablissement Français du Sang, Montpellier, France) by Ficoll-Hypaque density gradient centrifugation. $CD4^+$ T cells were purified (purity >95%) by negative selection from mononuclear cell preparations, using the RosetteSep procedure (StemCell Technologies, Vancouver, Canada), according to the manufacturer's instructions. The $CD4^+$ T cell clones used in this study were generated (Lecart et al., 2001) and maintained in culture (Yssel and Spits, 2002) as described previously.

To carry out a meaningful comparison between the different experimental conditions, the same two mAbs were used throughout the study: T cells (2×10^6 cells/ml) were stimulated with the anti-CD3 mAb SPV-T3b (IgG2a, Spits et al., 1983) and the anti-CD28 mAb L293 (IgG1, Testi and Lanier, 1989). Two modes of activation were used: stimulation of T cells with both mAbs, covalently linked onto magnetic beads (Expander Beads, Dynal, Oslo, Norway), was compared with a stimulation involving anti-CD3 mAb immobilized onto plastic tissue culture plates and soluble (1 μg/ml) anti-CD28 mAb (Umetsu et al., 1987; Levine et al., 1996). For coating onto plastic, anti-CD3 mAb was incubated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at a concentration of 10 μg/ml in PBS. After incubation at 4 °C for 24 h, the wells were washed twice with PBS and once with culture medium prior to the addition of the cells. When using plate-bound anti-CD3 mAb for stimulation, plates were spun for 2 min at $190 \times g$ prior to incubation in order to enhance interaction of the cells with the mAb (Yssel and Spits, 2002). T cells were harvested after incubation for 24 h at 37 °C and 5% CO_2 for analysis by immunofluorescence and flow cytometry. Culture supernatants were harvested after 48 h of incubation for analysis by ELISA. Cultures of T cells that had been stimulated with bead-linked mAbs were passed over a magnetic particle separator prior to analysis in order to remove magnetic beads (see under Section 2.3). All experiments were carried out in Yssel's medium (Yssel et al., 1984), supplemented with 1% human AB^+ serum (Etablissement Français du Sang, Lyon, France).

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2.2. Immunofluorescence and flow cytometry

All immunofluorescence and flow cytometry procedures were carried out as described (Scheffold et al., 2001). The following (m)Abs were used: FITC-conjugated and non-conjugated anti-CD3 mAb B-B10 and anti-CD25 mAb (kindly provided by Dr. John Wijdenes, Diaclone Research, Besançon, France), a non-conjugated anti-CD69 mAb, a non-conjugated and FITC-conjugated IgG1 mAb as isotype-specific negative controls (purchased from Becton-Dickinson, San Jose, CA) and a FITC-labelled goat anti-mouse Ig Ab (Caltag, Burlingame, CA). Results were analyzed on a FACSCalibur® flow cytometer, calibrated with FITC-coupled Callibrite® beads according to the recommendations of the manufacturer and equipped with Cellquest software (Becton Dickinson). Settings of the flow cytometer for a typical experiment were as follows: Detector FSC, voltage E00, AmpGain 2.25, Mode Lin; Detector FCS: voltage E00, AmpGain 2.25, Mode Lin; Detector SSC, voltage 577, AmpGain 1.12, Mode Lin; Detector FL1: voltage 752, AmpGain 1.00, Mode Log.

2.3. Detection and removal of shed CD3/CD28 mAb complexes

The presence of shed CD3/CD28 mAb complexes in culture supernatants was measured by ELISA as follows: wells of a 96-well flat bottom plate were coated with a goat anti-mouse IgG (γ and L chain-specific) Ab (Caltech) at a concentration of 2 μg/ml for 2 h at 37 °C and washed with PBS, supplemented with 0.05% Tween. Culture supernatants to be tested were incubated for 1.5 h at room temperature and after washing, a goat anti-mouse Ig coupled to alkaline phosphatase (Dako, Trappes, France) was added at a dilution of 1:1000. After an incubation of 1 h at room temperature, wells were washed, substrate (Sigma 104, Sigma-Aldrich, L'isle d'Abeau Chesnes, France) was added and the absorbance measured at 450 nm in an ELISA plate reader (Dynatech, MR5000). As a standard, mouse IgG (Southern) was used. The detection limit of the assay was 5 ng/ml.

Shed bead-linked CD3/CD28 Ab complexes were removed by incubating culture supernatants with goat anti-mouse Ig-coated magnetic beads (Dynal) at 4 °C for 30 min in 2-ml vials on a rotating device, followed by placing the vials in a magnetic particle separator (Miltenyi Biotech, Paris, France). Culture supernatants from T cells stimulated with plate-bound mAbs were incubated with goat anti-mouse Ig linked magnetic beads (Dynal) and further treated as described above.

2.4. Analysis of cytokine production by ELISA

Two-hundred-thousand cloned human T cells were transferred to the flat bottom wells of a 96-well culture plate (Nunc) and stimulated as described under Section 2.1. After incubation of the cultures at 37 °C 5% CO_2 for 48 h, production of IL-4, IL-5, IL-10 and IFN-γ was analyzed by specific ELISA, as described previously (Pène et al., 1998; Lecart et al., 2001).

3. Results and discussion

3.1. Magnetic bead-linked and plate-bound anti-CD3/soluble anti-CD28 mAbs activate $CD4^+$ T cells to a similar extent

The concentration of magnetic bead-linked anti-CD3 and anti-CD28 mAbs required to induce optimal T cell activation was determined by analyzing the expression of CD25, as expression levels of this cell surface molecule correlate with the magnitude of activation. Maximal T cell activation was observed at a concentration of about 5 magnetic beads/cell, resulting in high levels of CD25 expression on more than 90% of purified $CD4^+$ T cells (Fig. 1). A bead/T cell ration of 10:1 yielded similar results (data not shown; Anne-Marie-Rasmussen, Dynal Biotech, Oslo, unpublished results). The frequency of $CD25^+$ T cells stimulated with bead-linked mAbs was generally somewhat higher as compared to that following stimulation with plate-bound anti-CD3/soluble anti-CD28 mAbs (Fig. 1). Furthermore, cytometric analysis showed that the magnitude of activation following the different stimulation conditions also correlated with the side scatter (SSC) and forward scatter (FSC) properties, representing granularity and size, respectively, of the cells. Similar results were obtained with $CD4^+$ T cell clones (Fig. 1 and other results not shown).

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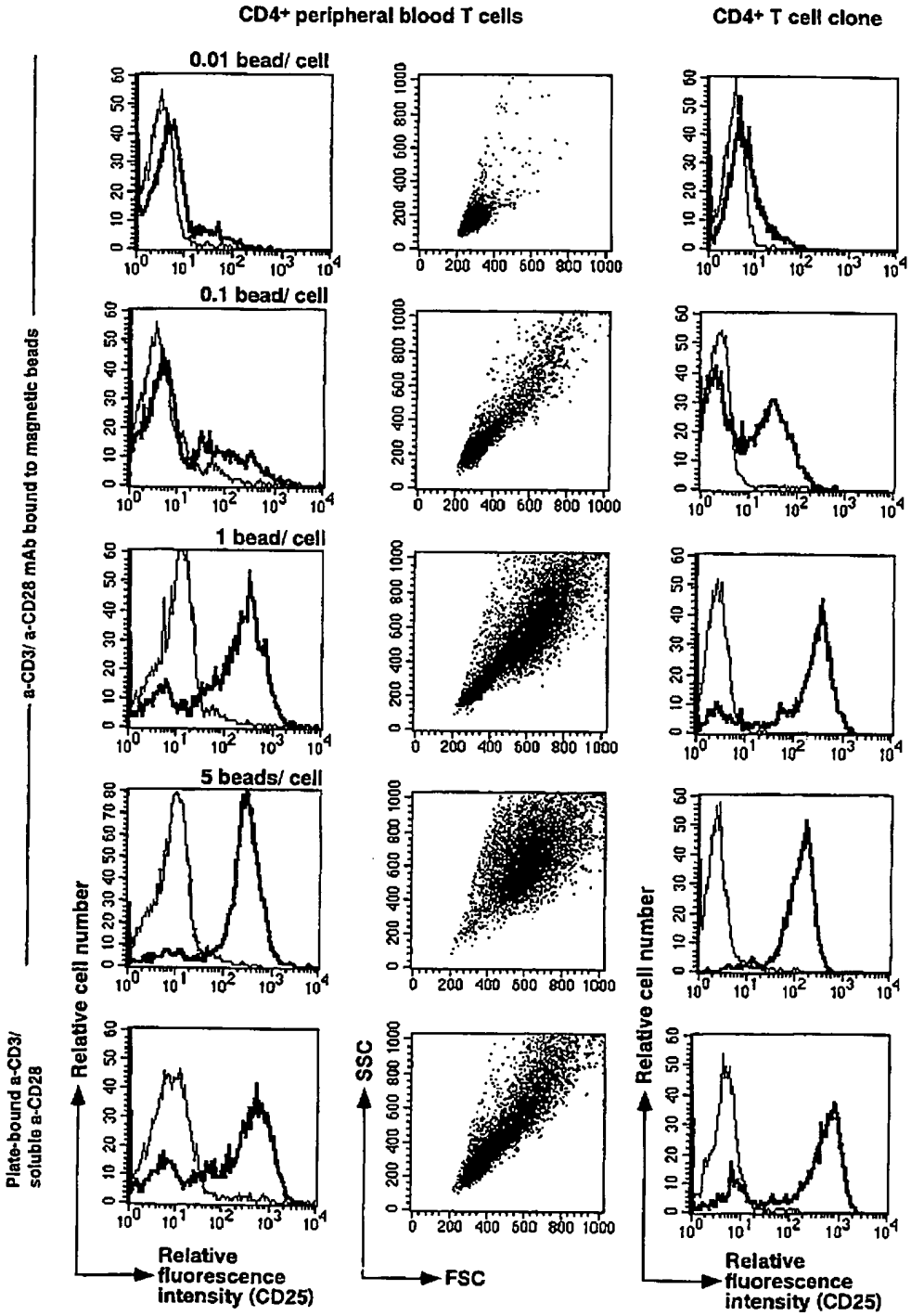

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The capacity of both modes of activation to induce the production of cytokines was also tested on $CD4^+$ T cell clones. Results from experiments with two T cell clones with a Th2-like cytokine production profile, permitting the analysis of several cytokines, are shown in Table 1. Similar to the results with respect to CD25 expression, levels of cytokine production correlated with the amounts of magnetic bead-linked mAbs used to stimulate the cells, with a plateau of about 5 beads/cell. Stimulation of the cells with a higher bead-to-cell ratio generally resulted in somewhat better sustained, although not statistically significant, cytokine production levels (results not shown). Cytokine production levels were comparable to those following stimulation with plate-bound anti-CD3/soluble anti-CD28 mAbs. Moreover, cytokine production profiles did not differ between both stimulation conditions. Together, these results show that mAbs directed at CD3 and CD28, and covalently linked to magnetic beads, are able to induce appropriate T cell activation.

Table 1
Activation-inducing capacity of magnetic bead-linked anti-CD3/CD28 mAb, as compared to plate-bound anti-CD3/soluble anti-CD28 mAbs

Stimulation conditions	Cytokine production (ng/ml ± S.D.)[a]			
	IL-4	IL-5	IFN-γ	IL-10
BOY.JF157				
Medium	<0.05	<0.01	<0.01	<0.01
Immob-a-CD3/s a-CD28[b]	14.6 ± 3.9	9.5 ± 4.0	<0.01	5.9 ± 2.9
Bead-linked aCD3/CD28[c]				
0.01 bead/cell	1.5 ± 1.3	2.6 ± 1.3	<0.01	2.2 ± 1.2
0.1 bead/cell	7.5 ± 3.4	11.0 ± 5.8	<0.01	4.9 ± 1.8
1 bead/cell	10.1 ± 4.4	14.5 ± 4.1	<0.01	5.9 ± 2.8
5 beads/cell	12.7 ± 4.8	16.7 ± 3.4	<0.01	4.5 ± 2.4
PUE.F3.9				
Medium	<0.01	<0.01	<0.01	<0.01
Immob-a-CD3/s a-CD28[b]	6.4 ± 3.5	14.0 ± 4.1	4.0 ± 2.2	5.5 ± 1.9
bead-linked aCD3/CD28[c]				
0.01 bead/cell	1.4 ± 0.5	7.5 ± 2.2	0.3 ± 0.1	1.4 ± 0.2
0.1 bead/cell	5.8 ± 1.6	20.0 ± 3.5	1.8 ± 0.1	5.8 ± 1.3
1 bead/cell	8.2 ± 4.3	22.5 ± 4.0	4.3 ± 0.2	8.2 ± 2.6
5 beads/cell	9.8 ± 3.4	19.7 ± 5.1	3.7 ± 0.9	10.7 ± 4.8

[a] The Th2 T cell clone BOY.JF157 and the Th0/Th2 T cell clone PUEF.3.9 were stimulated with anti-CD3 from 3 independent experiments.

[b] Plate-bound SPV-T3b (10 μg/ml coating) and soluble L293 mAb (1 μg/ml).

[c] SPV-T3b and L293 mAbs cross-linked onto magnetic beads.

3.2. Stimulation of $CD4^+$ T cells with magnetic bead-linked anti-CD3 and anti-CD28 mAbs results in complete removal of CD3/CD28 mAb complexes from the cell surface

Interaction of certain cell surface molecules with specific mAbs may result in the down-regulation and shedding (or internalization) of the cell surface molecule/mAb complex (Antel et al., 1982; Reinherz et al., 1982, Telerman et al., 1987). We therefore determined levels of cell surface expression of CD3/CD28 mAb complexes on activated $CD4^+$ T cells following their stimulation with magnetic bead-linked mAbs or plate-bound anti-CD3/soluble anti-CD28 mAbs. Significant levels of such complexes were still present at the surface of T cells stimulated with plate-bound anti-CD3/soluble anti-CD28 mAbs, as detected by staining with an FITC-conjugated Ab specific for mouse Ig (Fig. 2A). In contrast, however, no CD3/CD28/mAb complex was detectable on T cells that had been stimulated with anti-CD3/CD28 mAb immobilized on magnetic beads, as shown in a representative experiment in Fig. 2B. From a methodological point of view, the absence of residual anti-CD3 and anti-CD28 mAbs on the cell surface permits subsequent staining of the T cells for the expression of other cell surface molecules by indirect immunofluorescence, as is shown for CD25 and CD69 in Fig. 2D.

Fig. 1. Expression of CD25 by $CD4^+$ peripheral blood T cells and T cell clones following stimulation with anti-CD3 and anti-CD28 mAbs. One million cells per milliliter of purified $CD4^+$ peripheral blood T cells or of Th2 cell clone BOY.JF157 were stimulated either with different concentrations of the anti-CD3 mAb SPV-T3b and anti-CD28 mAb L293, covalently linked to magnetic beads, or with SPV-T3b, immobilized to tissue culture plates and soluble L293, as described in Materials and methods. Expressions of CD25 and SSC (granularity)/FSC (size) were measured by immunofluorescence and/or flow cytometry. Histograms from cells stained with the FITC-conjugated anti-CD25 mAb B-B1 (bold histograms) are superimposed over those from an isotype-matched control mAb (thin histograms). The x and y axes represent fluorescence (four decade log scale) and relative cell number, respectively. Representative results from six independent experiments.

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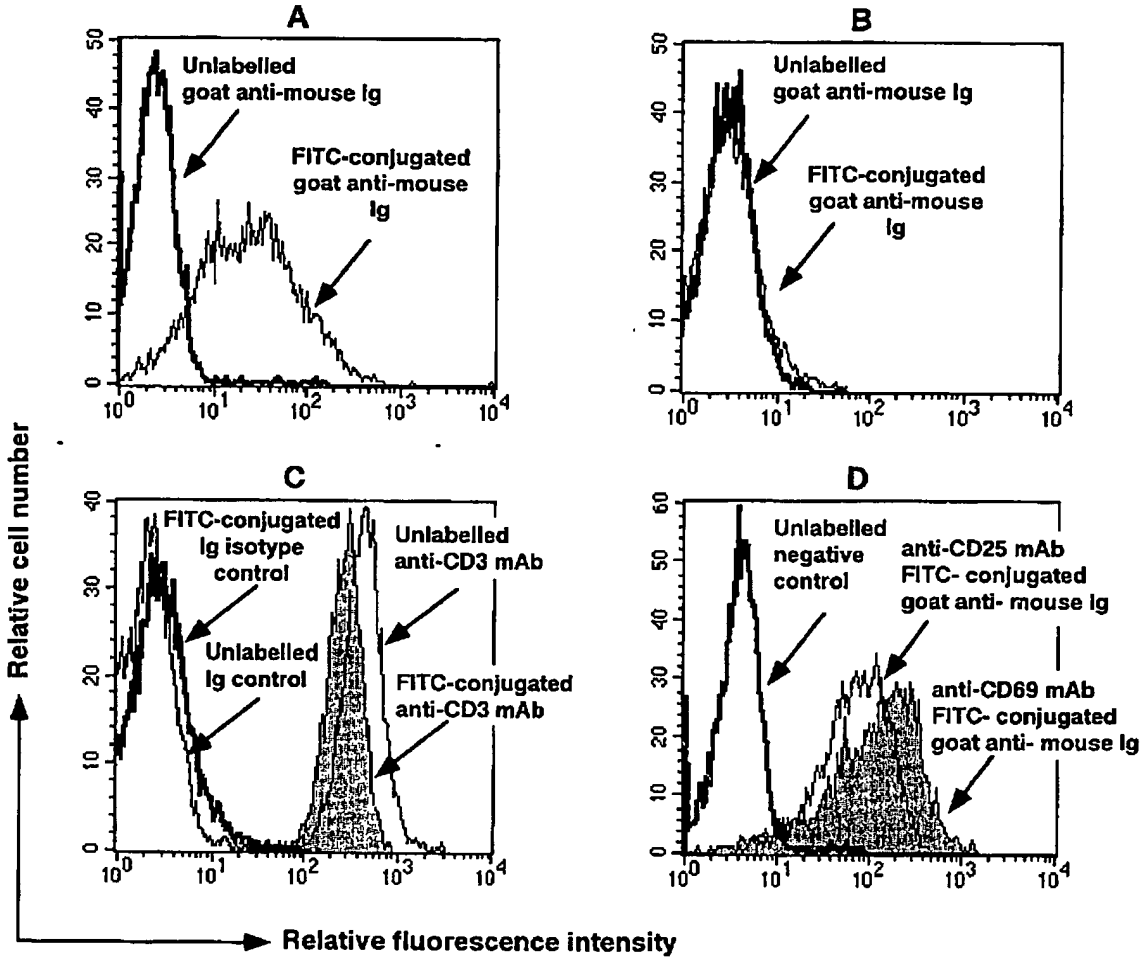

Fig. 2. Immunofluorescence staining pattern of a T cell clone with anti-CD3 and anti-CD28 mAbs. T cell clone BOY.JF157 (10^6 cells/ml) was stimulated either with plate-bound anti-CD3 and soluble anti-CD28 mAb (A) or with these mAbs linked onto magnetic beads (B, C, D). Staining conditions are indicated in the histograms. The x and y axes represent fluorescence (four decade log scale) and relative cell number, respectively. Representative results of two independent experiments.

The down-regulation of cell surface CD3, following binding of an anti-CD3 mAb (Antel et al., 1982; Reinherz et al., 1982), mimics the fate of the TCR following interaction with its physiologic ligand, since it has been shown by Valitutti et al. (1997) that TCR/CD3 complex expression is also down-regulated on T cells following stimulation with peptide-pulsed APC. This modulation from the cell surface is associated with an antigen dose-dependent decrease of the total cellular content of CD3-ε, and ζ chains. The time-course of the decrease in overall CD3 protein expression was found by these authors to overlap with the kinetics of TCR down-regulation and suggests a rapid degradation of internalized TCR–CD3 complexes (Valitutti et al., 1997).

It is of note that T cells, stimulated with anti-CD3 mAb, still expressed significant levels of CD3 as revealed by staining with a directly FITC-conjugated anti-CD3 mAb (Fig. 2C), indicating that only a fraction of the CD3 molecules, bound to the bead-linked anti-CD3 mAb, had been modulated from the cell surface. In the same vein, this observation also

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shows that only a few anti-CD3 mAbs are required to activate the cells. Indeed, Valitutti et al. (1995) have reported that a very small number of TCR molecules is involved in the interaction with specific antigen and APC, thereby transducing an activation signal via CD3. Taken together, the results presented here support the physiologic relevance of stimulation of T cells with anti-CD3 mAb-bound beads, which constitutes an alternative for the use of specific antigen because of the cumbersome and technically very demanding procedure of large-scale culture of human antigen-specific T cell lines.

Monocytes are able to engulf the type of magnetic beads used in the present study (Anne-Marie-Rasmussen, Dynal Biotech, personal communication). However, due to their size and physical properties, these beads, unlike anti-CD3 mAb/protein complexes, are not taken up by T cells and the modulation of these complexes from the cell surface is likely due to shedding, rather than their internalization. Indeed, high levels of CD3-anti-CD3 mAb complexes were detected in culture supernatants from T cells stimulated with mAb-linked magnetic beads. However, these complexes were also present in supernatants from T cells stimulated with anti-CD3 mAb immobilized onto a plastic tissue culture plate. This is somewhat unexpected since only very low quantities of soluble mAb could be detected in the culture medium in these wells already after 18 h of incubation at 37 °C in the absence of T cells (Table 2), indicating that once incubated on a plastic surface, anti-CD3 mAb remains relatively firmly attached. It seems, therefore, that interaction with T cell surface-expressed CD3 will strip the mAb off the tissue culture plate and that the CD3/anti-CD3 mAb complex is subsequently shed from the cell surface. The presence of such complexes in culture supernatants might interfere with functional assays which are based on proliferative responses of T cells, activated via immobilized anti-CD3 and CD28 mAb, as a read-out system. Yet, complexes of cell surface molecules and mAbs can easily be removed using a goat-anti mouse Ab coupled to magnetic beads (Table 2).

A major problem with the use of mAbs immobilized on plastic is the lack of quantification and reproducibility of the various methods used to coat the mAbs. Moreover, a mAb will adhere to the plastic surface with both its F_c portion and its variable F_{ab} region, whereas over time a number of the mAbs will detach and become soluble (Table 2). In this respect, the use of mAbs, linked to magnetic beads, allows optimization and standardization of cellular activation conditions, thus enhancing the reproducibility of the experiments. Taken together, the data presented here show that stimulation of T cells with magnetic bead-linked anti-CD3 and anti-CD28 mAb provides a physiologic mode of activation which, due to its capacity to completely remove the CD3/CD28 molecules from the cell surface, permits further analysis of cell surface molecules expressed on these cells by indirect immunofluorescence and flow cytometry. Finally, we should like to stress that this method has been successfully applied for the screening of T cell subpopulation-specific Ig-containing hybridoma supernatants in our laboratory (J. Pène and H, Yssel, unpublished data).

Table 2
Interaction of CD3 with plate-bound anti-CD3 mAbs results in the shedding of anti-CD3/mAb complexes into the culture supernatant

Conditions[a]	CD3/mAb protein concentration (ng/ml)	
	Exp 1	Exp 2
Medium	12	<5
BOY.JF157	107	79
BOY.JF161	134	230
After removal with goat anti-mouse Ab[b]		
Medium	<5	<5
BOY.JF157	<5	<5
BOY.JF161	<5	<5

[a] Wells of a 24-well tissue culture plate were coated with anti-CD3 mAb, as described in Materials and methods and incubated with culture medium, with the $CD4^-$ Th2 clone BOY.JF157 or the Tr1 clone BOY.JF161 (2×10^6 cells/ml). After 18 h of incubation, culture supernatants were harvested and levels of soluble anti-CD3 mAb/CD3 complexes were determined by ELISA.

[b] Culture supernatants were incubated with an Ig-specific goat anti-mouse Ab, linked to magnetic beads and treated as described in Materials and methods.

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